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Curtis Eugene Lanier

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**CHARACTERIZATION OF Na^+/H^+ EXCHANGER-3 (NHE3) IN THE GILLS OF
LONGHORN SCULPIN (*MYOXOCEPHALUS OCTODECEMSPINOSUS*).**

by

CURTIS E. LANIER

(Under the Direction of James B. Claiborne)

ABSTRACT

Acid-base regulation is a vital mechanism in homeostasis. Fish must maintain optimal physiological acid-base parameters during changes in an aquatic habitat. Environmental factors such as changes in salinity affect fish ability to maintain optimal blood plasma ion concentrations. Ion regulation influences the fish ability to maintain optimal acid-base balance. The Na^+/H^+ Exchanger-3 (NHE-3) has been shown to be the primary membrane ion transporter responsible for acid-base balance in the mammalian kidney. The objective of this study is to characterize NHE-3 in the gills of longhorn sculpin (*Myoxocephalus octodecimspinosus*).

INDEX WORDS: Acid-base balance, Na^+/H^+ Exchanger-3, Longhorn sculpin, Ion regulation

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LONGHORN SCULPIN (*MYOXOCEPHALUS OCTODECEMSPINOSUS*).**

by

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(B.S., Georgia Southern University, 2000)

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

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INTRODUCTION

The aquatic habitat challenges fish homeostatic regulation of internal pH, osmotic, and ion levels. Some of the challenges are the aqueous medium has a low O₂ solubility of ~7 ml/l or 3% that of air, high viscosity (800 times air) and a high density (60 times air) (Evans, *et. al.*, 2005). A high CO₂ solubility of the aqueous medium causes a rapid loss of CO₂ from the tissues since fish use a large gill ventilatory flow rate that creates a much lower internal blood plasma CO₂ concentration. The loss of CO₂ creates a much smaller blood to water CO₂ difference in partial pressure (1-4 mm Hg) as compared to terrestrial animals (30-45 mm Hg; Heisler, *et. al.*, 1989). The small difference in arterial blood to external water CO₂ partial pressure limits the regulation of blood plasma CO₂ by respiratory hyper- or hypoventilation, a mechanism used primarily by mammals (Woodbury, J. W., *et. al.*, 1965). As a consequence, the blood bicarbonate concentration of fishes is low (~4 mM compared with 24 mM in humans). The low bicarbonate concentration decreases the ability of fishes to regulate acid-base disturbances by respiratory compensation (Truchot, J. P., *et. al.*, 1987). Without a respiratory compensation mechanism for acid-base regulation, fish must use other mechanisms for transfer of nonvolatile acid-base equivalents with their aqueous environment (Claiborne, J. B., *et. al.*, 1998).

Fish ion transport and acid-base balance are mechanisms vital for maintaining optimal physiological pH in the face of both endogenous and exogenous changes (Claiborne, J. B., *et. al.*, 1997). Like the majority of organisms, fish are subject to endogenous formation of metabolites during metabolism that changes the internal physiological pH or ion concentration. Fish may also encounter changes exogenously,

which affect internal blood plasma pH and osmolarity due to inhabiting a rapidly fluctuating aquatic environment. Some exogenous stresses include hypoxia (low O₂), hypercapnia (high CO₂) and changes in external water salinity.

Freshwater teleosts face a volume load and salt loss, while marine teleost must deal with volume depletion and an increased salt load (Evans, D. H., *et. al.*, 1993). Observations into the different mechanisms of osmoregulation between fresh and seawater teleost have shown that seawater fish must drink large amounts of seawater while excreting a concentrated urine due to the marine teleost being hypoosmotic to seawater. Freshwater fish drink little water while excreting dilute urine due to fresh water fish being hyperosmotic to fresh water (Smith, H. W., 1930). Further studies using a euryhaline teleost showed that drinking of seawater was only necessary when external environmental osmotic concentrations were above that of the blood plasma.

A number of studies have observed the role of the kidneys in fish acid-base regulation and showed that the kidney has a small contribution to acid-base regulation in fishes (Heisler, N., 1993). This decreased role of acid-base regulation is not because the kidney is inefficient at acid-base regulation in fishes. The rate of renal transfer is about 0.007 to 0.025 (μmol ion transfer per μmol O₂ consumption), which is similar to mammals of 0.02 to 0.03. The kidney contribution is small because gill is more effective at ionic transfer due to the high rate of water flow across the gills.

Fish Gill Structure

There are several characteristics of fish gills that make them highly efficient at transfer of molecules between the internal and external environment. Gills have a large surface area and the epithelium is in direct contact with the external aquatic environment.

The rate of water flow across gill epithelium is around two orders of magnitude greater than the rate of urine flow from the kidney in fishes or in mammals (Janis, and Farmer, 1998). Therefore, molecules can be rapidly transferred between the organism and the environment without the need of large concentration gradients. Each branchial arch in fish contains multiple filaments, which are further subdivided into thousands of lamellae that are the sites of gas exchange (Evans, D. H., *et. al.*, 1999). The gill epithelium contains mucous cells, neuro-epithelial cells, pavement cells, mitochondrion-rich cells and accessory cells. The mitochondrion-rich cells or chloride cells have high metabolic activity for rapid ion transfer. The water that flows over the gill filaments is counter-current to the route of the blood that circulates the lamellae, maximizing gas, ionic, and osmotic gradients. This structural mechanism facilitates gas exchange, but also enhances net ionic and osmotic movements across the gill epithelia that the fish must counter to maintain osmotic homeostasis (Evans, D. H., 1999).

Membrane Ion Transport

Ionic gradients across the plasma membrane are regulated primarily by mechanisms of the lipid bilayer as a highly selective impermeable ion barrier with multiple ion transport proteins, channels, and pumps. In contrast to a high concentration gradient of Na^+ , intracellular pH is maintained at a neutral range (pH 7.2), which is much higher than that (~ 6.2) calculated by assuming that intra- and extracellular H^+ distribution follows its electrochemical gradient (Wakabayashi, S., *et al.*, 1997). NaCl secretion by teleost gills is by secondary active transport of Cl^- and passive transport of Na^+ utilizing the concentration gradient movement of Na^+ from aqueous marine environment (~ 500 mM Na^+) across the gill epithelium into the internal blood plasma (~ 150 mM Na^+)

(Figure 1). The driving mechanism for the active transport is basolateral Na^+, K^+ -ATPase, which keeps the intracellular Na^+ at low levels (50 mM) and intracellular K^+ levels high (Evans, D, *et. al.*, 2005). Since the gill epithelium has a low osmotic permeability, the Cl^- anion enters the cell via basolateral $\text{Na}, \text{K}, 2\text{Cl}$ co-transporter driven by sodium gradient from the internal blood plasma (150 mM) into the intracellular cytoplasm (50 mM). The intracellular Cl^- accumulates above its electrochemical equilibrium which allows exit passively at the apical membrane through CFTR type anion channels which facilitates the excretion of Na through tight junctions between gill epithelium (Evans, *et. al.*, 2005). The ability of marine fish to tolerate passive uptake Na is believed to facilitate apical Na/H exchanger export of intracellular H (Claiborne, 1998).

The Na^+/H^+ exchanger ion transport proteins use the energy stored in the Na^+ electroneutral gradients to decrease intracellular acidity by removal of excess H^+ . The Na^+/H^+ exchanger transfers 1 (Na^+) for 1 (H^+) across the cell membrane (Wakabayashi, S., 1997). The exchange reaction is reversible and driven by transmembrane chemical gradients for Na^+ and H^+ , without an input of metabolic energy such as ATP hydrolysis (Wakabayashi, 1997). The active excretion of H^+ causes an imbalance in the concentration of charged polar molecules in the cytosol allowing passive uptake of Na^+ across the cell membrane. Intracellular physiological conditions such as pH stimulates the ion exchange rate of Na^+/H^+ exchanger. This stimulation results from the intracellular pH dependence of the rate of exchange that allows the exchanger to respond to only small changes in intracellular pH (Wakabayashi, S., 1997). The cytoplasmic H^+ acts as an allosteric modifier of the exchange. The Na^+/H^+ exchanger (NHE) antiporter is believed to be the primary mechanism in ion and acid-base balance in saltwater and some fresh

water fish by the mechanism of acid excretion and salt uptake across the high-resistance tight gill epithelia (Claiborne, J. B., *et. al.*, 1999).

NHE Exchanger

The Na⁺/H⁺ exchanger (NHE) is one of the most highly studied mechanisms characterized for transport of ions and acid-base regulation. Historically, studies on NHE trace back to the chemiosmotic hypothesis (Mitchell, P., and Moyle, J., 1961). Mitchell investigated the importance of cation/H⁺ exchange and experimentally showed their presence in rat liver mitochondria. Ion exchange activity of NHE was first demonstrated in the bacterium *Streptococcus faecalis* (Harold, F.M. and D. Papineau, 1972). The mammalian Na⁺/H⁺ exchanger was then shown to function in brush border vesicles of rabbit kidney and small intestine (Murer, H., *et. al.*, 1976). Since these first investigations, the existence of this transporter has been described in many cell types in both prokaryotes and eukaryotes. In addition to pH regulation, the Na⁺/H⁺ exchanger is also involved in cell volume regulation (Yun, *et. al.* 1995).

Many NHE isoforms exist in both mammalian and non-mammalian systems. There are at least 11 different isoforms with NHE1-3 being the most frequently researched. Based on their primary structure, a similar membrane topology is seen among all isoforms, with a 10-12 transmembrane-spanning (TM) region at the N terminus domain and a large cytoplasmic region at the C terminus domain (Orlowski, J., and Grinstein, S., 1997). The N terminus domain is ~ 450 – 500 amino acid residues long and the C terminus domain is ~300 amino acid residues long (Zizak, M., 2000). The membrane-spanning segments M3-M12 show high homology between the various isoforms. Of these, M6 and M7 are the most highly conserved (95% identity) between

the TM regions of the NHE isoforms suggesting that this region participates in the transport of Na^+ and H^+ across the membrane. The NHEs vary in the 5' signal sequence and the cytosolic tail region consisting of ~300 amino acids.

In the mammals, NHE-1 is ubiquitous amongst cells and is located on the basolateral membrane in polarized cells serving a house keeping function of intracellular pH and volume regulation. The NHE-2 and NHE-3 are apically located in many epithelial tissues and believed to function in transepithelial Na^+ reabsorption and acid secretion. NHE-2 is mainly expressed in the gastrointestinal tract and is in its greatest concentration in the kidney proximal tubule, followed by small intestines and stomach (Wakabayashi, S., 1997).

NHE-1

The first isolated NHE cDNA clone was found to be a housekeeping gene, now known as human NHE-1. The gene for the human NHE-1 (NM003047) has a size of 70 kb, is composed of 12 exons and 11 introns (Miller, R.T., *et al.*, 1991), and is located on chromosome 1p35-36.1 (Matter, M.T., *et al.*, 1988). The mature human NHE-1 is a lipoprotein with a molecular mass of 105-120 kDa in SDS polyacrylamide gels (Fafournoux, P., *et al.*, 1994, Haworth, R.S., *et al.*, 1993, and Sardet, C., *et al.*, 1990). In some instances, a second band of 205-230 kDa is observed which increases in intensity when cells or membranes are exposed to cross-linking agents (Fafournoux, P., *et al.*, 1994 and Fliegel, L., *et al.*, 1993), suggesting that NHE-1 can exist as a homo-dimer. It is not known if the homo-dimer is the functional form. NHE-1 contains 6 external loops (ELS) with 5 of them except for the second loop, consisting of long extracellular stretches of 15-7 amino acids (Wakabayashi, *et al.* 1997). The first and fifth (ELS) are

very hydrophobic and contain two putative N-linked glycosylation sites (Asn-75 and Asn-370). In human NHE-1, only the Asn-5 (N75) amino acid residue is N-glycosylated (Counillon, L. *et al.*, 1994). Also, there is evidence of O-linked glycosylation (Counillon, L., 1994 and Sardet, C., *et al.*, 1989). The glycosylation is believed to affect the proper transport of the NHE-1 protein to the basolateral membrane (Conpaye-Gerard, B., *et al.*, 1996). Secondary structure analysis shows 10-12 (Borgese, F., *et al.* 1992, Reilly R.F., *et al.*, 1991, and Sardet, C., 1989) or 13 (Orlowski, J., *et al.*, 1992) transmembrane (TM) domains within the N-terminal portion of the molecule and a hydrophilic C-terminal tail. Truncation of the hydrophilic C-terminal tail of NHE-1 does not disrupt the ability to perform Na^+/H^+ exchange, suggesting that the N-terminal (TM) portion is the primary domain for transport (Wakabayashi, S., *et al.*, 1992).

The (K_m) of wild type transporters for extracellular sodium vary from 4.7 mmol/l for rat (Orlowski, J., *et al.*, 1993), over 13.9 mmol/l (Counillon, L., *et al.*, 1997) and 23 mmol/l (Touret, N., *et al.*, 2001), to 136 mmol/l (Wang, D., *et al.*, 1993) for the expressed human NHE-1. The reason for this large variation in K_m of expressed NHE1 for sodium is unknown.

Cysteine scanning analysis shows the accessibility of putative external loops (ELS) and internal loops (ILS) to a SH-reagent in intact and permeabilized cells expressing the human NHE-1 (Wakabayashi, S., *et al.*, 2000). These studies show IL2 connecting TM4 and TM5, and IL4 between TM8 and TM9 form hairpin structures embedded in the membrane and may play a role in Na^+ and H^+ transport. The previously predicted TM10 only touch the outer surface of the membrane (ELS). The cysteine scanning studies predict an additional TM, leading to a revised topology, model

consisting of 12-Trans membrane domains, 5 intracellular and 6 extra cellular loops, and with intracellular located N- and C-termini (Wakabayashi, S., 2000). The three-dimensional assembly of the transmembrane domains is not known.

NHE-2

The NHE-2 homolog was cloned from human AF073299 (Ghishan, F.K., *et al.*, 1995), rat NM012653 (Collins, J.F., *et al.*, and Wang, Z., *et al.*, 1993), and rabbit L13733 (Tse C-M, *et al.*, 1993) tissues. The cDNAs coded for proteins that had 812 (human; NP003039), 813 (rat; P48763), or 809 (rabbit; P50482) amino acids. The human NHE-2 gene is localized on chromosome 2q11.2 (Ghishan, F.K., *et al.*, 1995). The promoter/enhancer region of rat NHE-2 showed a number of regulatory elements including five glucocorticoid-responsive elements and one progesterone-responsive element (Muller, Y. L., *et al.*, 1998). This finding suggested that NHE-2 responds to the hypertonic conditions of kidney medulla and may serve to maintain cell volume by an increased influx of salt and water into the cells.

Northern blot studies revealed the presence of NHE-2 mRNA in human colon, small intestine, stomach, kidneys, liver, heart, testes, uterus, and adrenal glands (Ghishan, F.K., *et al.*, 1995). In rats, the highest signals were found in uterus and liver, lower ones in stomach, much less in jejunum and colon, and no signal in kidneys (Bookstein, C., *et al.*, 1997 and Collins, J.F., *et al.*, 1993). In the rabbit, the amounts of message decreased in the order of renal medulla and ascending colon > renal cortex and adrenal glands > descending colon > jejunum > ileum > duodenum (Collins, J.F., *et al.*, 1998). It has also been found in rat renal proximal tubules (Sun, A.M., *et al.*, 1997), ascending thin and thick limbs of Henle's loop and distal convoluted tubule (Chambrey, R., *et al.*, 1998).

Amino acids 731-777 in the cytoplasmic tail were found to be responsible for the apical sorting of rat NHE-2 (Chow, C.W., *et. al.*, 1999). Human, rat and rabbit NHE-2 contain one or more putative sites for N-glycosylation. Rabbit NHE-2 contains O-linked carbohydrates, the removal of which does not affect the function of the exchanger (Tse, C-M., *et. al.*, 1994). Secondary structure analysis shows 10 (human) (Ghishan, F.K., *et al.*, 1995), 11 (rat) (Tse, C-M., *et. al.*, 1993) and 12 (rabbit) (Tse, C-M., *et. al.*, 1993) transmembrane domains. The membrane topology of NHE-2 is unknown. Functional characteristics of extracellular sodium activated NHE-2 with Michealis-Menten kinetics is in agreement with a single cation-binding site (Levine, S.A., *et. al.*, 1993 and Tse, C-M., *et. al.*, 1994). The K_m values for sodium are 14.4 mmol/l for expressed rabbit NHE2 (Yun, C.H.C., *et. al.*, 1993), and 33.1 (Honda, T., *et. al.*, 1993) or 50 mmol/l (Yu, F.H., *et. al.*, 1993) for rat NHE-2. Activation by intracellular protons shows cooperative kinetics (Levine, S.A., *et. al.*, 1993 and Yu, F.H., *et. al.*, 1993), consistent with the presence of an H^+ -binding modifier site. The NHE-2 amiloride affinity is high with K_i values of 1 mmol/l (rabbit) when compared to other homologs (Tse, C-M., *et. al.*, 1993).

NHE-3

The NHE-3 isoform, which is the most important NHE for intestinal and renal salt absorption, was first cloned in human NM04174 (Brant, S.R., *et. al.*, 1995), rat M85300 (Orlowski, J., *et. al.*, 1992), and rabbit M87007 (Tse, C-M., *et. al.*, 1992) tissues. The protein consists of 831 (rat P26433) and 832 (rabbit P26432) amino acids. The coding region of the rat NHE-3 isoform has 17 exons. The gene for human NHE-3 is found on chromosome 5p15.3 (Brant, S.R., *et. al.*, 1993). In situ hybridization detected NHE-3 mRNA in villus cells of human ileum>jejunum> colon (Hoogerwerf, W.A., *et. al.*, 1996).

Northern blots of rat tissues have shown NHE-3 mRNA in the proximal colon> small intestine>kidneys>stomach (Orlowski, J., *et. al.* 1992). RT-PCR analysis detected NHE-3 mRNA in rat thick ascending loop of Henle's (TALH) (Borensztein, C., *et. al.*, 1995). The strongest NHE-3 mRNA signal in Northern blots from rabbit tissues are found in the kidney cortex, and weaker signals are found in the ileum and colon and even lower mRNA signal in jejunum and renal medulla (Tse, C-M., *et. al.*, 1992). In immunocytochemical studies, human NHE-3 is found in the brush-border membrane of duodenum (Repishti, M., *et. al.*, 2001), and jejunum through to the rectum (Hoogerwerf, W.A., *et. al.*, 1996). Although rabbits show a similar NHE-3 distribution, there is no detection in the descending colon (Hoogerwerf, W.A., *et. al.*, 1996). In rat kidney, NHE-3 protein is either detected in the brush-border membrane of cells in the proximal tubule segments (Biemesderfer, D., *et. al.*) S1 and S2, but not S3 segments (Amemiya, M., *et. al.* 1995), or in S1, S2 and S3 segments and also in the apical membrane of thick ascending limb cells (Biemesderfer, D., *et. al.*, 1997). In high resolution of images of rat kidney proximal tubules, the protein is not only present in the microvilli, but also in endosomes, which may form a reservoir of NHE-3 (Biemesderfer, D., *et. al.*, 1997).

Like other NHEs, The NHE-3 isoform has a N-terminal membrane domain and a long C-terminal cytoplasmic loop. The secondary structure of NHE-3 is unknown. However, the NHE-3 has 11-13 predicted transmembrane domains for the N-terminal part of the protein (Orlowski, J., *et. al.*, 1992, Zizak, M., *et. al.*, 2000). In Western blots, Human NHE-3 shows a sharp band at 82 kDa (Counillon, L., *et. al.*, 1994), rat, 80 kDa (Biemesderfer, D., *et. al.* 1993), 83-45 kDa (Azuma, K. K., *et. al.*, 1996) and 87 kDa (Amemiya, M., *et. al.*, 1995); rabbit, 95-100 kDa (Soleimani, M., *et. al.* 1994); dog, 83-

90 kDa (Soleimani, M., *et. al.*, 1994). Deglycosylation of NHE-3 does not change its mobility in SDS polyacrylamide gels, suggesting that at least the rat NHE-3 is not glycosylated (Counillon, L., *et. al.*, 1994). Thus similar to NHE-1 and NHE-2, glycosylation does not seem to be important for function. Extracellular sodium activated NHE-3 with hyperbolic Michaelis-Menten kinetics, indicating the presence of a single cation-binding site (Levine, S. A., *et. al.*, 1993). The K_m for Na^+ is 10 mmol/l and is far below extra cellular Na^+ concentration (Orlowski, J., *et. al.*, 1993). Hence, NHE-3 is saturated with Na^+ and operates in the forward direction. One study shows the pK for half-maximal activation of the human NHE-3 is 7.1, whereas that for NHE-1 was 6.75. The high affinity for intracellular protons keeps human NHE-3 active at a physiological cell pH of 7.2 (Yoshitomi, K., *et. al.*, 1984). It is not known whether species or experimental differences are the cause of this difference in proton affinity. The affinity of NHE-3 to amiloride is low compared to NHE-1 and NHE-2. The difference between the high (NHE-1) and low (NHE-3) affinity for amiloride and its analogs persisted when the cytoplasm loops were exchanged, suggesting affinity is determined by the N-terminal domain (Wakabayashi, S., *et. al.*, 1995). It has been shown that the N-terminal TM4 and TM9 domains of NHE-3 determine the low affinity of NHE-3 to inhibitors like amiloride (Yun, *et al.*, 1995).

Mice lacking a functional NHE-3 show a decrease of proximal tubular bicarbonate absorption by 54% (Wang, T., *et. al.*, 1999). The results from the NHE-3 knockout mice fits with amiloride or its analogs inhibition seen in rat renal tubules which suggest that up to 68% of bicarbonate absorption are due to the operation of the Na^+/H^+ exchanger (Chan, Y. L., *et. al.*, 1981, and Preisig, P., *et. al.*, 1987). Therefore, more than

half of proximal tubular bicarbonate absorption can be attributed to the function of NHE-3. The knockouts had a significantly reduced blood pressure (Lorenz, J. N., *et. al.*, 1999), and died within four days on a low salt diet, showing the importance of NHE-3 on salt and water balance. Whereas in the kidneys the reduced proximal salt absorption was efficiently counterbalanced by a decrease in glomerular filtration rate via the tubular glomerular feedback, salt and water uptake from the intestine was lowered, and diarrhea occurred in the knockout mice (Lorenz, J. N., *et. al.*, 1999). Also, the NHE-2 that is expressed all over the small and large intestines could not take over salt and water absorption in the absence of NHE-3 in the knockout mice.

NHE-4

The Na⁺/H⁺ exchanger 4 isoform (NHE-4) was first cloned from rat NM173098 (Orlowski, J., *et. al.*, 1992). The gene for human NHE-4 (NP001011552) is located on the chromosome 2q11-q12 next to NHE-2 (Diamanduros personal communication). The NHE-4 protein has 798 amino acids with 13 predicted transmembrane domains in the N-terminal domain and contains the C-terminal cytoplasmic tail. Monoclonal antibodies against NHE-4 react in Western blot with a 65-70 kDa band. The molecular weight of rat NHE-4 in SDS polyacrylamide gel electrophoresis is smaller than the molecular weight calculated by amino acid sequence, suggesting NHE-4 is not glycosylated.

Tissue distribution analysis by Northern blot reveals mRNA for NHE-4 in rat stomach > small and large intestine > kidneys, uterus, and skeletal muscle (Orlowski, J., *et. al.*, 1992). Within the kidneys, mRNA was found in the cortex (Bookstein, C., *et. al.*, 1996). Reverse transcriptase-PCR on rat and mouse kidneys revealed the presence of NHE-4 mRNA in inner medullar collecting ducts. A polyclonal antibody against NHE-4

was used for immunohistochemistry in rat kidney. Simultaneous use of antibodies to Tamm-Horsfall glycoprotein and aquaporin-2 or -3 permitted identification of thick ascending limbs and collecting ducts, respectively (Paillard, M., *et. al.*, 2001). The results indicate that NHE-4 is highly expressed in basolateral membranes of thick ascending limb and distal convoluted tubule, whereas collecting ducts from cortex to inner medulla and proximal tubules showed weaker basolateral NHE-4 expression. Western blot analysis of NHE-4 in membrane fractions prepared from the inner stripe of the outer medulla revealed the presence of a 95-kDa protein that was located in basolateral membrane vesicles isolated from medullary thick ascending limbs.

Transfected full-length NHE-4 is quiescent in exchanger deficient fibroblast when acid-loaded at isoosmolarity but is activated when acid-loaded under hypoosmolar conditions (Bookstein, *et. al.*, 1994). The activation of NHE-4 in hypoosmotic conditions suggests that this isoform has a specific role in the kidney in balance of cell volume in response to extreme cell shrinkage.

NHE-5

The NHE-5 isoform was first cloned from a human genomic library using NHE-2 as a probe (Klanke, C. A., *et. al.*, 1995). The gene for human NHE-5 (NM 004594) is located on chromosome 16q22.1. The NHE-5 has a size of 1.8 kb with 16 exons and 15 introns in its coding region, and codes for a protein with 896 amino acids (Baird, N. R., *et. al.*, 1998). The location of NHE-5 is found in the brain, testes, spleen and skeletal muscle. Immunocytochemical localization of NHE-5 and its functional characterization has not been reported.

NHE6-NHE11

Further investigation of the NHE transporter family has found other homologs that are divergent from the plasmalemmal-type NHEs. Studies have shown that there is organelle associated NHE6-9 homologs that are found ubiquitously within all tissues serving a house keeping function (Orlowski, J., and Grinstein, S., 2004). The NHE-6 homolog was first cloned in human (NM006359) using yeast NHE-2 probe. The human NHE-6 protein (Q92581) has 669 amino acids arranged in 12 transmembrane domains. NHE6 is a mitochondrial Na^+/H^+ exchanger. The NHE-7 isoform shows 70% homology with NHE-6 differing mostly in the N- and C-terminus portion of the protein. The NHE-7 localizes primarily in the *trans*-Golgi network with associated endosomes. The NHE-8 homolog is only 28% homologous to NHE6-9, which suggest that NHE-8 is the most evolutionarily distant homolog. Also, the c-terminal hydrophilic domain is shorter (~100 amino acid residues) as compare to other homologs (150-190 amino acid residues) suggest that NHE-8 is functionally different than the other organelle NHE isoforms (Nakamura, N., *et. al.*, 2005). The NHE-8 homolog is found in higher amounts in the skeletal muscle and kidney in the mid- to *trans*-Golgi network. The NHE-9 isoform is closely homologous to NHE-6 and NHE-7. NHE-9 is found in the post-Golgi network and is distributed ubiquitously in the tissue.

Other studies have detected NHE isoforms that are not grouped with NHE1-5 or NHE6-9. The NHE-10 homolog has been shown to be involved in pH regulation in sperm (Wang, D., *et. al.*, 2003). Studies have shown that NHE-10-null mice were completely infertile with severely diminished sperm motility. The human NHE-10 gene is located on chromosome 3q13.2 and has a predicted topology of 14 transmembrane

domains. Whole-genome shotgun assembly (WGSA) of the human genome generated at Celera in 2001 has detected a NHE-11 gene (Istrail, S., *et. al.*, 2004). The NHE-11 isoform is located on chromosome 1q25.1. The human NHE-11 tissue distribution and topology have not been reported.

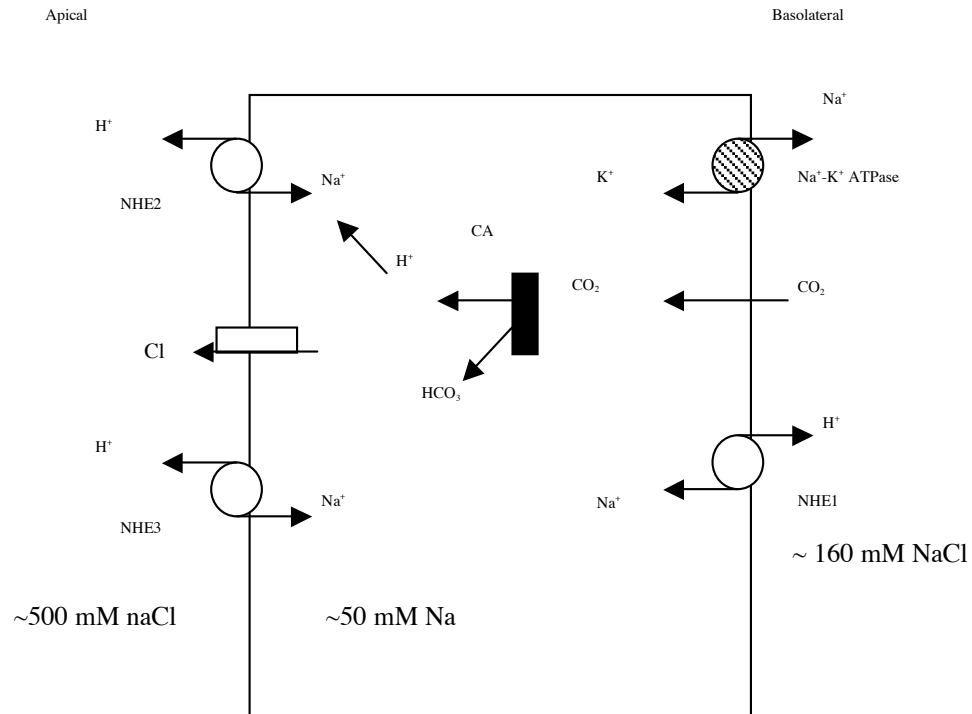


Figure 1: Proposed model of ion transport and acid-base regulation in salt water fish gill. The H^+ and HCO_3^- are generated in the cell due to hydration CO_2 by carbonic anhydrase enzyme. An apical Na^+/H^+ antiporter exchanges intracellular H^+ for extracellular Na^+ at near the rate as an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger. Modified from Claiborne (1997, 1998).

Objective

The NHE exchanger is believed to facilitate NaCl absorption and H^+ extrusion in both marine and freshwater fishes (Figure 1; Claiborne, J. B., 2002). Western blotting using heterologous antibodies to screen for expression of NHE-1 and NHE-3 in gills of

an agnathan (*Myxine glutinosa*) and an elasmobranchs (*Raja erinacea*), and teleost (*Fundulus heteroclitus*; AY818824) showed positive NHE-1 bands in gills from the agnathan and the elasmobranchs and positive bands for NHE-3 antibodies in the gills of the elasmobranchs and the teleost (Choe, K. P., *et. al.*, 2002). Other studies have identified mRNA of NHE-1 and NHE-2 like isoforms in longhorn sculpin (*Myoxocephalus octodecimspinosus*; AF159879), mummichog (*Fundulus heteroclitus*; AY868824) and hagfish (*Myxine glutinosa*) using RT-PCR with primers generated from putative membrane spanning domain of the human NHE-1 exchanger (Claiborne, J. B., 1999 and Edwards, S. L., *et. al.*, 2001).

Edwards *et. al.* (2005) induced acidosis in the mummichog (*F. heteroclitus*) by ambient hypercapnia (1% CO₂) and observed an increase in net H⁺ excretion. Fish were pre-adapted to fresh water (FW), brackish (iso-osmotic; BW) and seawater (SW). Both FW and SW adapted mummichog were tested for NHE protein expression using mammalian NHE antibodies. NHE immunoreactive proteins were detected in gill membrane homogenates from both fish groups. Hypercapnia induced a threefold increase in gill NHE-2 like protein expression in FW fish, but SW adapted fish showed inconsistent NHE-3 like expression. There was no change in NHE-1 levels in FW fish. In contrast, SW fish showed a significant increase of expression in both NHE-1 and NHE-3 like proteins following hypercapnia but low expression of the NHE-2 protein. The study showed that different homologs of NHE are possibly expressed depending on the ambient salinity. Net H⁺ transfers during acidosis may be partially driven by increased expression of specific NHE transporters (Edwards, S. L., *et. al.*, 2005).

Molecular cloning using degenerative primers and RT-PCR of gill cDNA library of the Osorezan dace (*Tribolodon hakonensis*; AB065466) identified a 4.0 kb NHE3 sequence (Hirata, T., *et. al.*, 2003). The dace grows in the extremely acidic (pH 3.4-3.8) Lake Osorezan and migrates to neutral streams for spawning. Lake Osorezan is located in the Shimokita Peninsula in the northern part of Honshu, the mainland of Japan, which is a freshwater environment. Real-time PCR was used to show a significant increase of NHE-3 in dace exposed to high acid with increasing time of exposure (Hirata, *et. al.*, 2003). Also, Analysis of the *Fugu rubripes* genome (Elgar, *et al.*, 1999), which became available in October of 2001, has shown sequences that have a high homology to mammalian NHE-3.

Another study used degenerate primers and RT-PCR to identify a partial sequence for both NHE-2 (AY626249) and NHE-3 (AY62650) homologs from the gills of the euryhaline Atlantic stingray (*Dasyatis sabina*) (Choe, K. P., *et. al.*, 2005). Real-time PCR was then used to show that mRNA expression of the NHE-3 homolog increased when stingrays were transferred to low salinities. Hypercapnia did not stimulate an increase of mRNA expression of NHE-3 using real-time PCR. Expression of the NHE-2 isoform did not change with either treatment. Rapid amplification of cDNA ends (RACE) was used to determine the complete sequence of NHE-3. The 2,744-base pair cDNA includes a coding region for an 837-amino acid protein that is 70% identical to human NHE-3. Polyclonal antibodies specific for the carboxyl tail of the putative stingray NHE-3 labeled the apical membranes of Na⁺/K⁺-ATPase-rich epithelial cells, and acclimation to freshwater caused a redistribution of labeling in the gills. This study identified the first NHE-3 cloned from an elasmobranch and was the first to demonstrate

an increase in gill NHE-3 expression during acclimation to low salinities, suggesting that NHE-3 can absorb Na^+ from low ionic environments (Choe, *et. al.*, 2005).

To date there is no report of NHE-3 in marine teleost gill epithelia. NHE-3 is the most highly expressed isoform in mammalian kidney proximal tubules and is expressed in other fish for Na^+/Cl^- reabsorption and H^+ excretion, my hypothesis is that NHE-3 is present in marine teleost gill epithelia. Characterization of a NHE-3 isoform in a marine teleost will allow novel insight into the role of NHE-3 in gill epithelial transport for ion and acid-base regulation.

MATERIALS AND METHODS

Animal Holding Conditions

Long-horn sculpin (*Myoxocephalus octodecimspinosus*) were collected by commercial fisherman from Mount Desert Island Frenchman Bay and maintained in aquariums with running seawater at (12-15°C) until needed. Sculpin were restricted from eating for one week before being used for experimentation. Individual fish were removed from the primary holding tank with some being immediately weighed and sacrificed while others were anesthetized with MS-222 and weighed for cannulation. Sacrificing of fish involved a brain/spinal pithing method of quickly cutting the spinal cord and pithing the brain area of the fish. Gill filaments were dissected and placed in ice cold buffer (250 mM sucrose, 1 mM EDTA, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µg/ml PMSF, and 30 mM Tris-Cl at pH 7.4) and cells disrupted with a polytron homogenizer (on ice) for immediate RNA isolation or the surgically removed gill filaments were stored in RNAlater media (Ambion) at -20°C according to manufactures protocol.

Cannulation was performed by surgically inserting P50 size tubing into the sculpin peritoneal cavity. To prevent the fish from removing the tubing, it was sutured to the body wall. Cannulated sculpin were placed into individual plexiglass containers with aerated running seawater at (12-15°C) and acclimated for 1 day before injections.

Sculpin were injected via the implanted cannula with water or 0.02 N HCl according to calculated conversion by weight. Sculpin were sacrificed in 30 min, 2hr. and 4hr. intervals using brain/spinal pithing. Gill filaments were stored using methods described above.

RNA Isolation

Isolation of total RNA from gill homogenates of long-horned sculpin was performed using TRI reagent (Molecular Research Center, Inc.) method of extraction. Extracted tissue was homogenized in 14 ml round-bottom Falcon 352059 tubes in 1ml TRI Reagent per 50-100mg tissue. The homogenate was stored at room temperature for 5-15 minutes then transferred in 1 ml aliquots to 1.5 ml microcentrifuge tubes. A 1-Bromo-3-chloropropane (BCP) solution (BCP; 10.0 ml per 1.0 ml TRI reagent used) was then added. The mixture was incubated at room temperature for 2-15 minutes and then centrifuged at 12,000 g for 15 minutes at 4°C. The procedure separated the mixture into three layers: RNA (top, aqueous phase). DNA (interphase). And protein (lower, organic phase). The aqueous phase containing RNA, was transferred to a new 1.5 ml tube being careful not to disturb the white DNA interphase. The RNA was then precipitated with 0.5 ml isopropanol for each ml TRI reagent used in the initial homogenization.

The sample was incubated at room temperature for 5-10 minutes and centrifuged at 12,000 g for 8 minutes at 4°C. The supernatant was removed from resulting RNA pellet. The RNA pellet was then washed with 75% ethanol and centrifuge at 7,500 g for 5 minutes at 4°C. The resulting RNA was quantified and checked for purity using UV spectrophotometry at 260/280 nm and the integrity verified by agarose gel electrophoresis (1.3% agarose gel using a MOPS/formaldehyde buffer system) and ethidium bromide staining (Appendix).

RNA Analysis

Methods of RNA analysis included formaldehyde gel electrophoresis and spectrophotometer analysis (Biorad). Glassware was sterilized by heating in an oven at

275 °C for RNase elimination. All solutions were made RNase free by incubation overnight at 37°C with 0.1% diethylpyrocarbonate [(DEPC; Sigma, 1 (ml) to ddH₂O 1 (Liter))] and autoclaved for 1 hour. All equipment and utensils were made RNase free by washing with 20% SDS and rinsing with DEPC treated dH₂O. Molecular grade RNase free agarose (Fisher) was used to make a 1.3% RNA formaldehyde agarose gel for gel electrophoresis (Appendix B). Isolated RNA was mixed (1 µg/µl) with 10 µl of 2X RNA loading buffer into sterile 1.5 µl microcentrifuge tubes (Roche). The RNA samples and 2.0 µl per lane of RNA marker (0.24-9.49 kb RNA ladder; Invitrogen) were denatured at 65°C for 10 minutes in water bath and quick frozen on ice for 2 minutes before loading into gel wells for gel electrophoresis. Electrophoresis was performed at ~60.0 V for 3.0 – 4.0 hours depending on speed of sample traveling through the gel. Gels were photographed (FisherBiotech camera; Fisher Scientific) over ultraviolet (UV) light (UV Transilluminator; UVP. Inc.). Denaturing RNA formaldehyde gel allows the size of rRNA and integrity of total RNA to be determined using RNA size marker. The size of rRNA and integrity of total RNA is needed for further Northern blotting and cDNA synthesis.

Next, RNA samples were slowly thawed on ice, heated for 10-15 minutes at 65°C and immediately put on ice for spectrophotometer analysis. The total RNA concentration of each sample was determined by spectrophotometer analysis at 230 nm, 260 nm, and 280 nm since pure single-stranded RNA optical density at 260 nm and 1 cm light path length equals 40 µg/ml. The total RNA concentration was calculated by multiplying the mean optical density of the dilute RNA sample, the 1µl: 1000 µl dilution factor, and by 40 µg/ml for final unit measurement in µg/µl. For examination of isolated total RNA

sample carbohydrate and protein contamination, ratios 260/230 for carbohydrates and 260/280 for proteins were observed. Both ratios should be between 1.8 and 2.0 for clean RNA.

DNase Digestion of RNA Samples

To remove genomic DNA contamination from RNA samples for cDNA synthesis, samples were treated with DNase enzyme (Promega Corporation). The reaction mixture included: 1-2 µg total RNA, 1.0 µl 10X reaction buffer (200 mM Tris-HCl pH 8.4, 20mM MgCl. 2 and 500 mM KCl), 1.0 µl amplification grade DNase I (1U/µl), Sterile dH₂O up to total volume of 10.0 µl into 0.5 µl microcentrifuge tubes. The reaction mixture was incubated for 37°C for 30 minutes. Next, 0.1 µl of 25 mM EDTA was added to stop the reaction. The RNA was then isolated from the solution with TRI reagent using the protocol described earlier for total RNA isolation.

Degenerative Primer Design

Mac Vector 7.0 software (Oxford Molecular Ltd.) was used to develop degenerate forward and reverse primers from NHE-3 (Table 1) that were homologous to the conserved regions of Osorezan dace *Tribolodon hakonensis* (BAB83083) and pufferfish *Fugu rubries* (NC_004299) cDNA sequences (National Center for Biotechnology Information, NCBI). Degenerative primers were designed with 20-22 bp and G-C rich at the 3' end to prevent primer dimer formation between primer pairs. Also, the degenerative primers of a set were designed to have similar annealing temperatures.

Reverse Transcription cDNA Synthesis

Reverse transcription was performed on sculpin total RNA using the Superscript[™] II Kit (Invitrogen) according to manufactures protocol (Appendix A). Reverse

transcription utilized an avian Rnase H- reverse transcriptase and is designed to have higher thermal stability, and produce high yield and more full-length cDNA transcripts. Sculpin total RNA was heated for 10 minutes at 65°C and put on ice to remove secondary structure. The reverse transcription reaction cycle was one cycle of: 50 minutes at 42°C, 10 minutes at 72°C, In order to check for genomic DNA amplification a negative and positive superscript TM II reaction was performed. An amount of 0.5 µg/µl total sculpin RNA was used in the reverse transcription reaction.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification using degenerative NHE-3 primers on sculpin cDNA was performed using a FastStart *taq* polymerase kit (Roche) and a Thermo Hybaid thermocycler machine in a 50-µl reaction. The components were mixed and centrifuged briefly. The PCR cycle used an initial denaturation at 95°C for 5 minutes, 35 cycles of 1 minute each denaturing at 95°C, annealing from 52°C , elongation at 72°C and a final one step elongation at 72°C for 10 minutes. After completion of PCR, the products were mixed with 1.0 µl of 5X loading buffer and electrophoresis performed on a 1% agarose gel (Appendix). If there was significant background smear or multiple DNA bands in the range of expected size, the PCR product was used as the DNA template for Nested PCR using the internal primer sets. If the Nested PCR products were of expected size the samples were prepared for DNA sequencing.

Agarose Gel Electrophoresis

PCR products were visualized using gel electrophoresis through 1% agarose (Fisher Scientific) gel stained with ethidium bromide (~1 µl/200-250 mg agarose). A 1X

TBE (Tris-borate-EDTA) or TAE (Tris-acetate-EDTA) was used as a gel electrophoresis running buffer (Sambrook et al., 1989). A 6X loading dye (Promega) was used to load PCR samples. A 1kb ladder (Promega) was electrophoresed along with PCR sample DNA in separate wells of the gel. The voltage used for electrophoresis was usually set at approximately 90-100 V for 25-45 minutes dependant on gel size and dimensions. Once the DNA traveled 75% of gel distance the gel was viewed and photographed over UV light transilluminator.

Cloning of PCR Product

The PCR product DNA that showed the appropriate size upon gel electrophoresis and UV visualization was ligated into plasmid for cloning using the Invitrogen TOPO TA cloning system according to the manufactures procedures (Appendix C). The TOPO TA cloning system is a convenient system for the cloning of PCR products. The PCR product was ligated into an TOPO TA cloning kit (Invitrogen) plasmid vector for sequencing and the plasmids transformed using TOPO 10F' competent cells (Invitrogen). The TOPO TA Cloning sequencing kit uses pCR 4-TOPO plasmid that is specifically designed to clone *Taq* polymerase-generated PCR products for sequencing. *Taq* polymerase has a nontemplate-dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in the kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* Virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent

bond between the 3' phosphate of the cleaved strand and tyrosyl residue (Tyr274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can then be attached by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuan, 1994). TOPO[®] Cloning utilizes this reaction to efficiently clone PCR products.

The pCR[®]4-TOPO vector allows direct selection of recombinants via disruption of a lethal *E. coli* gene, *ccdB* (Bernard and Couturier, 1992; Bernard *et al.*, 1994; Bernard *et al.*, 1993). The vector contains the *ccdB* gene fused to the C-terminus of the LacZ α fragment. Ligation of a PCR product disrupts expression of the *lacZ α -ccdB* gene fusion permitting growth of only positive recombinants upon transformation in TOP10F' cells. Cells that contain nonrecombinant vector are killed upon plating.

The ligation procedure included mixing of each PCR product with TOPO kit components for a total reaction of 6 μ l each. The ligation reaction mixture was incubated for 5 minutes at room temperature of 22-23°C in 0.5 μ l microcentrifuge tubes. After incubation the ligation reaction was centrifuged briefly. Next 2 μ l of each ligation reaction was added to individual 5 minute thawed vials of TOPO 10F' competent *E. coli* cells. The cells were mixed gently by flicking sides of vials and incubated on ice for 30 minutes. Next the cells were heat-shocked in a water bath for 30 seconds at 42°C to allow plasmid entry through the cell membrane. After heating the cells were immediately transferred to ice and 250 μ l of room temperature SOC medium (Invitrogen). The liquid cell culture was incubated at 37°C for 1 hour at 200 rpm. Once the incubation was complete 10-50 μ l of each cell culture transformation was spread onto 37 °C prewarmed kanamycin (50 μ l) selective Luria-Bertani (LB) agar plates and incubated overnight at

37 °C. The pCR 4-TOPO plasmid contains a kanamycin resistance gene which when grown on antibiotic kanamycin-treated LB Agar plates eliminates growth of general bacteria not resistant to kanamycin or TOPO 10F' cells that do not contain the plasmid. For each transformation, several isolated colonies were selected and the cells were further amplified in LB broth containing 50 µg/µl Kanamycin (Gibco BRL;). Individual colonies were picked using a single sterile 100 µl pipette tip that is placed into approximately 5.0 ml of broth in a 15.0 ml Falcon tube. The tubes of broth were incubated at 37°C overnight with shaking at 220 rpm to ensure adequate mixing of the nutrients and oxygen. Stock plates of streaked individual colonies on plates and 1.5 microcentrifuge tube glycerol stocks (250 µl sterile 50% glycerol and 750 µl culture) were made for positive cultures for future analysis. The LB broth incubated transformations were used for mini-prep plasmid purification and sequencing.

Plasmid Purification

An alkaline lysis preparation was performed to extract the plasmid DNA from each clone using High Pure Plasmid Isolation kit (Roche Applied Science) according to manufactures instructions (Appendix C). Alkaline lysis releases plasmid DNA from bacteria. Bond plasmid DNA is purified in a series of rapid washing and spinning steps to remove contaminating bacterial components. Finally, low salt elution releases the DNA from the glass fibers. This method eliminates the need for organic solvent extractions and DNA precipitation, allowing for rapid purification of many samples simultaneously.

The initial step of the procedure involved centrifugation of 0.5-1.5 ml of the overnight transformed *E. coli* culture in 1.5 ml microcentrifuge tube at 9000 rpm for 30

seconds and 4 °C for sedimentation of a bacterial pellet. The resulting cell pellets each were resuspended in 250 µl of suspension buffer (1XTE buffer: 25 ml, 50 mM Tris-HCl, 10 mM EDTA pH 8 and 1 mg RNase A.). The suspension buffer provided a stable environment for the *E. coli* cells and appropriate pH for enzymatic activity of the RNase A enzyme for denaturing of bacterial RNA to eliminate further downstream RNA contamination in purified plasmid DNA sample. The cells were resuspended into suspension buffer solution by gently flicking the tube in order to make sure the bacterial chromosome was not dislodged from the cell membrane so that there would be no downstream contamination of bacterial chromosomal DNA in the purified plasmid isolate. In order to break up the bacterial cell membrane that would cause the release of intercellular components, a volume of 250 µl lysis buffer (25 ml, 0.2 M NaOH, 1% SDS) was added to each cell suspension. The solution was inverted a few times to mix the reagents. Next the solution was incubated at room temperature for 5 minutes. In order to precipitate out large pieces of chromosomal DNA and excess proteins, the pH was neutralized to 7.4 by the addition of 350 µl of chilled Binding buffer (25 ml, 4 M guanidine HCl, 0.5 M K⁺-Acetate, pH 4.8). The solution was gently mixed by inverting and incubated on ice for 5 minutes. Once the incubation was done the solution was centrifuged for 1 minute at 14,000 rpm. Following centrifugation a white precipitate containing the intracellular debris is formed on the sides of the tubes. The High pure filter tubes are placed in individual collecting tubes. The isolated plasmid DNA containing supernatant is pipetted into 700 µl polypropylene High Pure glass fiber filter tubes and the tubes are centrifuge for 1 minute at 14,000 rpm. The flow through was discarded from the collecting tube. After centrifugation, a volume of 500 µl Washing buffer I (33

ml, 5 M guanidine HCL, 20 mM Tris-HCL, pH 6.6 final concentration after addition of 20 ml ethanol) is added to the filter tube and the tube is centrifuged for 1 minute at 13,000 rpm. The flow through was discarded. A second volume of 700 µl Washing buffer II (10 ml, 20 mM NaCl, 2 mM Tris-HCL, pH 7.5 final concentration after addition of 40 ethanol) was added to High pure filter tube and the tube centrifuged for 1 minute at 13,000 rpm. The flow through containing collection tube was discarded and a 1.5 ml microcentrifuge tube was added onto the High Pure filter tube. Purified plasmid DNA is recovered in 1.5 ml microcentrifuge tube by addition of 100 µl of Elution buffer (30 ml, 10 mM Tris-HCL, pH 8.5) and the tube centrifuge for 1 minute at 13,000 rpm. The resulting solution of purified plasmid DNA is next quantified by spectrophotometer density analysis for restriction digest analysis and DNA sequencing.

Plasmid DNA Analysis

The total plasmid DNA concentration of each sample was determined by spectrophotometer analysis at 230 nm, 260 nm, and 280 nm since pure double-stranded DNA optical density at 260 nm and 1 cm light path length equals 50 µg/ml. The total DNA concentration was calculated by multiplying the mean optical density of the dilute plasmid DNA sample, the 1µl: 1000 µl dilution factor, and by 50 µg/ml for final unit measurement in µg/µl. For examination of purified plasmid DNA sample carbohydrate and protein contamination, ratios 260/230 for carbohydrates and 260/280 for proteins were observed. Both ratios should be between 1.8 and 2.0 for clean DNA. Once the concentrations of the plasmid DNA was determined the samples were ready for restriction digest and DNA sequencing.

Restriction digest

Restriction enzyme digestion was performed using an *EcoRI* (Invitrogen) restriction enzyme to ensure that the appropriate size insert was present in all isolated plasmids. The pCR4-TOPO plasmid contains *EcoRI* restriction sites flanking either side of the insert position that allows the restriction enzyme to cut in front and behind the location of the plasmid insert. To perform the restriction digest, a volume of 1 µl sample purified plasmid DNA was added to the *EcoRI* kit components for a total volume of 20 µl in a 0.5 ml microcentrifuge tube. The reaction mixture was vortex and centrifuged briefly. The reaction was incubated for 1 hour at 37°C in a water bath. A volume of 4 µl 10X DNA Loading buffer (20% glycerol, 0.1 M disodium EDTA pH, 1.0% SDS, and 0.25% bromphenol blue) for a total reaction mixture of 24 µl was added to enzyme digestion reaction. The reaction tube was vortex and centrifuged briefly. The samples were electrophoresis on a 1.0 % agarose gel (Appendix D) and visualized with UV light. Two distinct bands are detected with UV light of approximately 3.5 kb which is the cut pCR4-TOPO plasmid and a smaller less bright band that should be the same size as the original cloned PCR product.

DNA Sequencing

The plasmid DNA was sent to Mount Desert Island Biological Laboratory Sequencing Facility for nucleotide sequencing. The procedure involved the appropriate amount of plasmid DNA depending on the DNA concentration being added to sterile distilled water for a total volume of 22 µl. Next, a volume of 1 µl each of pCR4-TOPO plasmid M13 forward and M13 reverse sequencing primers were added to the sequence

reaction for a final volume of 24 μ l. Sequence analysis compared homology of the PCR fragment with other known NHE-3 sequences.

RACE Primer Design

Once some sequence of the sculpin NHE-3 gene was known, a set of gene specific primers (GSPs) homologous to the sculpin NHE-3 cDNA fragment was designed for a positive control PCR and for performing rapid amplification of cDNA ends (RACE) PCR (Appendix). The RACE PCR was performed using a GeneRacer kit (Invitrogen). The GSPs were used in conjunction with GeneRacer primers in the kit to amplify the remaining 5' end and 3' end of the sculpin NHE-3 cDNA (Figures 2a-f). One pair of GSPs was designed for the positive PCR. Two other pair of GSPs was designed for the RACE PCR. In most cases, one round is sufficient to generate a gene-specific RACE PCR product. However, if there was not a distinct RACE PCR product or an observed high background existed in the first RACE PCR then the second pair of nested sculpin NHE-3 GSPs was used for nested RACE PCR. GSPs were designed as nested primers for a second round of RACE PCR to get a more distinct band with low background sculpin NHE-3 PCR product. The nested RACE PCR was performed with the GeneRacer nested primers provided in the kit and the nested sculpin NHE-3 GSPs. The GSPs were designed with 50-70% GC content to obtain a high annealing temperature, 23-28 nucleotides in length to increase specificity of binding, low GC content at 3' ends to minimize extension by DNA polymerase at non-target sites (no more than two G or c residues in the last five bases), no self-complementary sequences within the primer or no sequence complementary to the primers supplied in the kit especially at the 3' end, annealing temperature near 72°C to increase specificity of the PCR and ability to perform

touchdown PCR. Touchdown PCR and high annealing temperatures increase the primer binding specificity and reduce non-specific amplification. The GSPs were designed as close to the sculpin NHE-3 cDNA fragment ends as possible to minimize the size of the RACE PCR product. Also, the nested GSPs were designed far enough from the original GSP so that there was a distinction between sizes of the original and nested RACE PCR product.

RACE RNA Modification

The GeneRacer technique is based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) method, and results in the selective ligation of an RNA oligonucleotide to the 5' ends of decapped mRNA using T4 RNA ligase (Maruyama and Dugano, 1994; Schaefer, 19994). Sculpin total RNA is treated with calf intestinal phosphatase (CIP) to remove the 5' phosphates (Figure 2a). This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer RNA Oligo. CIP has no effect on full-length, capped mRNA. The RNA dephosphorylation reaction procedure involved adding 1-5 µg total RNA to GeneRacer kit components for a total volume of 10 µl reaction mixture on ice in a 1.5 ml microcentrifuge tube. The reaction tube was vortex and centrifuged briefly. The dephosphorylation reaction was incubated at 50 °C for 1 hour in a water bath. After incubation the reaction tube was centrifuged briefly and placed on ice. To precipitate RNA, a volume of 90 µl DEPC water and 100 µl phenol: chloroform was added to the reaction tube. The tube was vortexed vigorously for 30 seconds and centrifuged at 14,000 rpm for 5 minutes at room temperature. After centrifugation the aqueous (top) phase (~100 µl) containing RNA was transferred to a new 0.5 ml microcentrifuge tube.

The Next a volume of 2 μ l 10 mg/ml mussel glycogen, 10 μ l 3 M sodium acetate at pH 5.2 and 220 μ l 95% ethanol was added to the isolated RNA solution. The mixture was vortexed and centrifuged briefly. The mixture was incubated at -80°C on dry ice for 10 minutes. This step caused precipitation of unwanted salts or remaining contaminants from isolated RNA solution. To pellet the RNA, isolated RNA solution was centrifuged

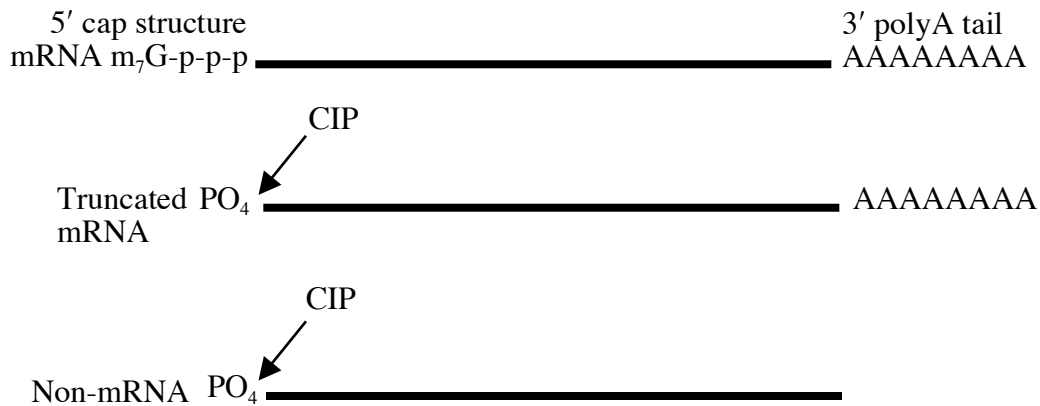


Figure 2: Calf intestinal phosphatase (CIP) removes the 5' phosphate from RNA. This eliminates truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer™ RNA Oligo. Note: CIP has no effect on full-length capped, capped mRNA.

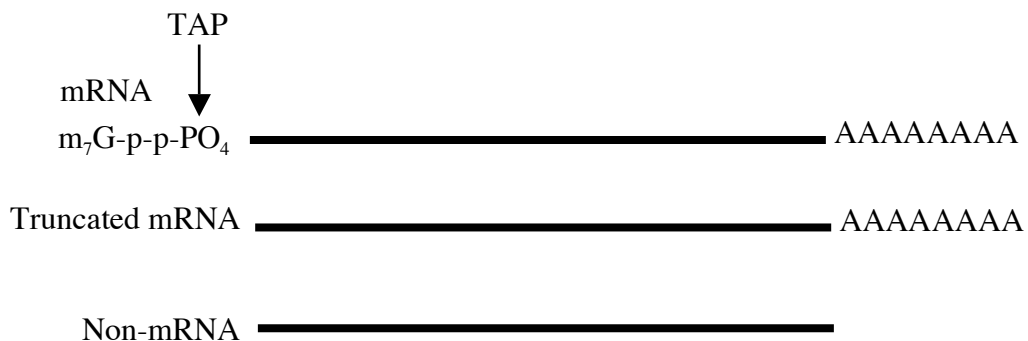


Figure 3: Dephosphorylated RNA was treated with tobacco acid pyrophosphatase (TAP) to remove the 5' cap structure from intact, full-length mRNA. This treatment leaves a 5' phosphate required for ligation to the GeneRacer™ RNA Oligo.

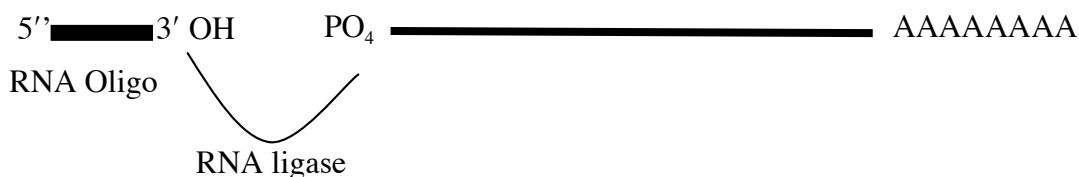


Figure 4: The GeneRacer™ RNA Oligo was ligated to the 5' end of the mRNA using T4 RNA ligase enzyme. The GeneRacer™ RNA Oligo will provide a known priming site for GeneRacer™ PCR primers after the mRNA is transcribed into cDNA.

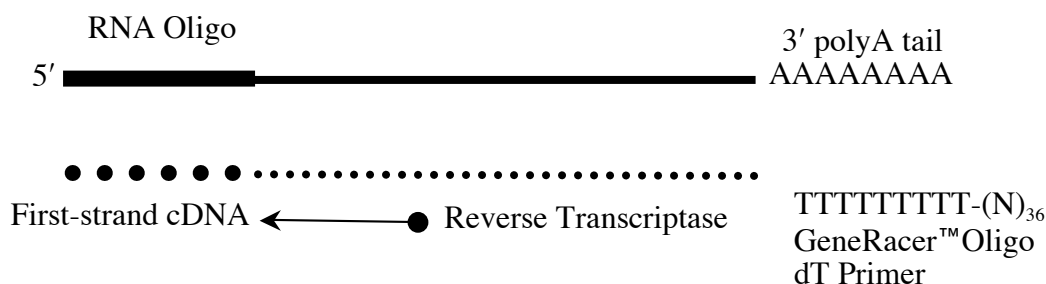


Figure 5: The ligated mRNA is reverse-transcribed using the Superscript™ II RT and the GeneRacer™ Oligo dT primer to create RACE-ready first-strand cDNA with known priming sites at the 5' and 3' ends.

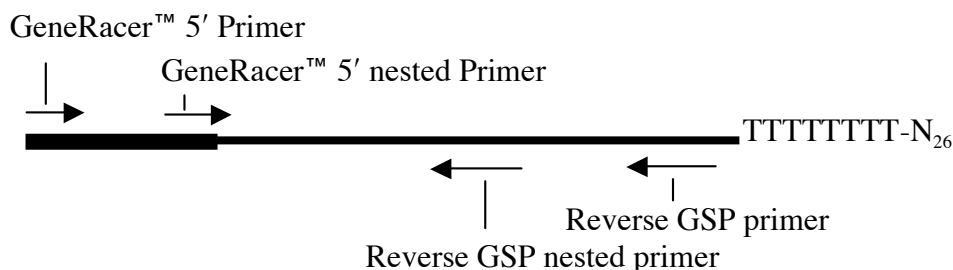


Figure 6: To obtain 5' ends, the first-strand cDNA was amplified using a reverse gene-specific primer (Reverse GSP) and the GeneRacer™ 5' Primer (homologous to the GeneRacer™ RNA Oligo). Only mRNA that has the Gene Racer™ RNA Oligo ligated to the 5' end and is completely reverse-transcribed will be amplified using PCR. If needed, an additional PCR with was performed with nested primers.

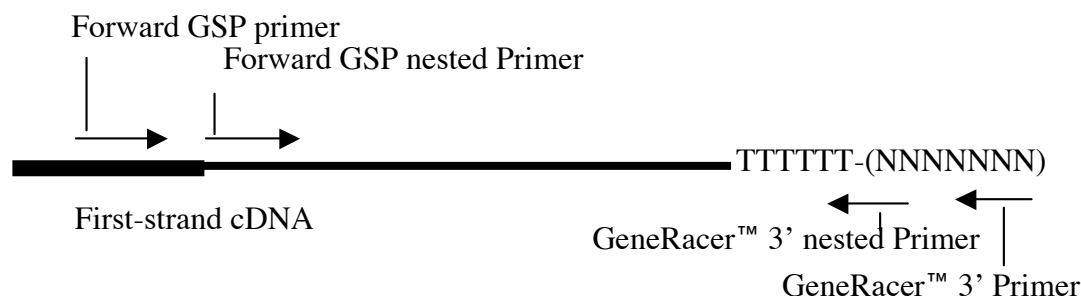


Figure 7: To obtain 3', the first-strand cDNA was amplified using a forward gene-specific primer (Forward GSP) and the GeneRacer™ 3' Primer (homologous to the GeneRacer™ Oligo dT Primer). Only mRNA that has a poly A tail and is completely reversed transcribed will be amplified using PCR. If needed, an additional PCR was performed with nested primers.

at 14,000 rpm for 20 minutes at 4 °C. After centrifugation, contaminate containing supernatant was pipetted from the tube and discarded leaving only the RNA pellet. The pellet was washed of any remaining contaminates by alcohol precipitation. A volume of 500 µl 70 % ethanol was added to the RNA pellet. The RNA was resuspended by inverting several times and vortexing briefly. The resuspended RNA solution was centrifuged at 14,000 rpm for 2 minutes at 4 °C. After centrifugation, the ethanol was carefully pipetted off the resulting RNA pellet. The RNA pellet was air-dried on ice for 2 minutes. The RNA pellet was resuspended into 7 µl of DEPC treated water and placed on ice.

After dephosphorylating and precipitating the RNA, the 5'cap structure from full-length mRNA must be removed (Figure 2b). This procedure involved mixing the 7 µl of dephosphorylated RNA solution with 5' cap removing Generacer kit components (Figure ???). The mRNA cap removing reaction was incubated at 37°C for 1 hour. After incubation the reaction tube was centrifuged briefly and placed on ice for 2 minutes. A

second phenol-chloroform RNA extraction and RNA alcohol precipitation was performed described earlier for purification of total RNA from other constituents. The resulting modified total RNA pellet was resuspended in 7 µl of DEPC treated water for further modification.

Further modification of the RNA included ligation of Generacer 5' forward primer complimentary RNA oligo sequence onto decapped full-length mRNA by adding generacer ligation components with 7 µl isolated modified total RNA in the 1.5 ml vial containing the lyophilized Generacer RNA Oligo (0.25 µg) (Table 1). The reaction was mixed by pipetting the solution up and down several times and centrifuged briefly. The reaction was incubated at 37°C for 1 hour. After incubation, the reaction was centrifuged briefly and place on ice. The modified RNA was extracted by phenol chloroform extraction and ethanol precipitation described previously. The purified RNA was resuspended in 10 µl. DEPC treated H₂O further reverse transcription cDNA synthesis.

RACE cDNA Synthesis

Reverse transcription (RT) of Sculpin mRNA using a reverse transcriptase enzyme was used for synthesis of Sculpin cDNA described previously. The Generacer kit uses an avian Rnase H⁻ Thermoscript RT enzyme for synthesis of cDNA. Thermoscript allows cDNA synthesis from RNA samples containing high G-C nucleotide content or large amount of secondary structure that can hinder movement of the RT enzyme along the mRNA strand during cDNA synthesis

RACE PCR

Gene specific primers (GSPs) developed from the detected sculpin NHE-3

sequence gene were synthesized for performing rapid amplification of cDNA ends (RACE) PCR using a GeneRacer kit (Invitrogen). The GSPs were used in conjunction with GeneRacer primers in the kit to amplify the remaining 5' and 3' end of the sculpin NHE-3 gene (Table 1). Two pair of GSPs homologous to the sculpin NHE3 cDNA fragment were designed with 50-70% GC content to obtain a high annealing temperature, 23-28 nucleotides in length to increase specificity of binding, low GC content at 3' ends to minimize extension by DNA polymerase at non-target sites (no more than two G or c residues in the last five bases), no self-complementary sequences within the primer or no sequence complementary to the primers supplied in the kit especially at the 3' end, annealing temperature near 72°C to increase specificity of the PCR and ability to perform touchdown PCR. Touchdown PCR and high annealing temperatures increase the primer binding specificity and reduce non-specific amplification.

The modified Sculpin cDNA was mixed in 50 ul 5' reaction and 3' reaction (Appendix). The PCR reaction was prepared as previously described. Touchdown PCR was used for amplification of Sculpin PCR product. The initial denaturing step was performed at 95°C for 5 min. Next, a 5 cycle 95°C denaturation for 30 sec and 72°C elongation for 3 min performed. Next, a 5 cycle 95°C denaturation for 30 sec and 68°C elongation for 3 min were performed.

Northern Blot

RNA formaldehyde gels were prepared as described previously. The RNA was visualized using UV light and the gels were photographed with camera. The photograph was later used to compare the distance of bond probe to the distance of the RNA marker bands seen on the photograph using a ruler. After electrophoresis, the gels were rinsed in

ice cold 500 ml H₂O for 45 minutes to remove the formaldehyde in the denaturing gel.

The formaldehyde in the gel interferes with the RNA capillary transfer mechanism in the Northern blot.

The gel was blotted overnight to transfer RNA to a positively charged nylon membrane (Millipore) by capillary action mechanism according to Sambrook *et al* (1989). The transfer buffer used was 10 TAE (Appendix). Several filter papers (Fisher Scientific) were stacked onto a plastic support, which was used for wicking the buffer from the reservoir in a rectangular Rubbermaid container. Bubbles were removed from under the filter paper by rolling a 15 ml falcon tube across it. The gel was placed upside down onto the filter paper and a corner of the gel was cut off for orientation. All bubbles were removed from beneath the gel by rolling with 15 ml falcon tube. The nylon membrane is cut to approximate dimensions of gel with some overhang at the top of the gel and submerged in 10X TAE for 20 minutes. The nylon membrane is placed on top the gel and a corresponding corner cut off. The top of the gel location was marked on the nylon membrane with a lead pencil for later measurement of RNA band sizes. A 15 ml falcon tube was used to remove the bubbles from under the nylon membrane. Parafilm strips were placed along the edges of the nylon membrane to prevent hindrance of the capillary action mechanism. Next, two filter paper were cut to the exact dimensions of the nylon membrane and submerged in 1X TAE for 2 minutes and placed on top of the nylon membrane. Next, 1-2 inches of paper towel was cut to exact dimensions of the filter paper and is placed on top of the filter paper. A glass plate was place on top of the paper towel for support of a heavy object. The heavy object was placed on top of the

glass plate to ensure a tight connection between the layers of paper for sufficient absorption of buffer.

The RNA was allowed to transfer for 12-24 hours depending on speed of wicking effect during capillary transfer. After the transfer of RNA, all absorbent paper was discarded and a lead pencil was used to mark the locations of the wells of the gel onto the nylon membrane. The location of the wells makes it easier to measure the RNA band sizes. The nylon membrane was removed from the gel. The gel was visualized under UV light. No RNA should be visible in the gel with complete capillary transfer to nylon membrane. The RNA was bonded to the nylon membrane using a UV cross linker. Also, RNA should be visible on the nylon membrane with exposure to UV light during UV cross-linking. The membrane was washed with gentle shaking in 5X TAE for 10 minutes to remove any excess agarose particles. The membrane was placed between two pieces of sterile filter paper and dried at 80 °C for 2 hours or until the membrane is completely dried which further binds the RNA to the membrane.

Two northern blot detection methods were used for quantification of NHE-3 in sculpin total RNA. The first method used labeling of a sculpin NHE-3 DNA probe with Digoxigenin (DIG) PCR probe synthesis kit (Roche) according to manufactures instructions. Ultrasensitive hybridization buffer (Ultrasens™; Ambion ®) solution from was used for hybridization of Sculpin NHE-3 DNA probe to complimentary mRNA on the nylon membrane (Millipore). The nylon membrane was rinsed in 100 ml 2X TAE for 20 minutes to recalibrate the membrane. The Ultrasens™ solution was warmed to 68°C for 15 minutes to reconstitute compounds in the solution. The Ultrasens™ was cooled down to 47°C in hybridization oven. A volume of 10 ml of Ultrasens™ was added to the

membrane in the hybridization oven cylindrical chamber as quickly as possible as to prevent drying out of the nylon membrane. Drying of recalibrated membrane causes background in later exposure of the membrane to x-ray film. Prehybridization was carried out at 47 °C for 1 hour. Prehybridization of the nylon membrane allows the Ultrahyb™ solution's blocking reagent to bind to non RNA bonded areas on the membrane preventing nonspecific binding of the DNA probe which will bind to the nylon membrane if not blocked. The DIG-labeled probe was denatured (5-25ng/ml hybridization solution) by boiling in water bath for 5 minutes and rapidly cooled on ice. Once prehybridization is complete, a total volume of 50 ul probe was added to the membrane submerged hybridization solution. The hybridization was performed at 47°C for 16-24 hours overnight.

Once hybridization was done, the nylon membrane was washed several times for removal of nonspecific probe hybridization. The first step involved two stringency washes of the membrane in 50 ml prewarmed 47°C 2X SSC, 1% (w/v) SDS at 47°C for 30 minutes per wash. Next, the membrane is washed twice more in 50 ml prewarmed 47 °C 2X SSC, 0.5 % (w/v) SDS () at 47°C for 30 minutes per wash. The stringency pre-wash was removed. Next, the probed membrane is washed using 1X washing buffer for 30 minutes at room temperature with agitation using a bench top shaker according to manufactures protocol (). Once washing is complete, the wash buffer is removed. The membrane is incubated at room temperature for 30 minutes in 1X blocking solution (100 ml) with agitation (). The blocking solution blocks the anti-DIG-AP antibody from binding to areas of the nylon membrane that are not probed. The blocking solution is removed. The membrane is incubated for 30 minutes in 20 ml antibody solution (ix)

containing anti-DIG-AP antibody with agitation. The antibody solution was removed. The membrane was washed twice for 15 minutes in 100 ml washing buffer at roomed temperature with agitation to remove excess blocking solution. Once washing of the membrane is complete, the probed membrane is removed from the hybridization canister with sterile forceps and the excess wash on the membrane was removed by touching one corner of the membrane to a sterile filter paper. The membrane was placed in a precut hybridization bag (Roche). The membrane was equilibrated for 3 minutes in 20 ml detection buffer (). The CDP-Star enzyme substrate (0.5 to 1 ml) () was applied to the membrane with DNA/RNA side facing up on the hybridization bag and heat sealed. The membrane bag was wrapped in aluminum foil and incubated at room temperature for 10 minutes. Once incubation was complete the membrane bag was place in developing cassette with X-ray film for overnight exposure. Once exposed, the film was removed from the cassette in the darkroom. While in the darkroom, film is submerged into 300 ml of 1:5 developer (Fisher) and tap water until developed (1-2 min). The developed film was submerged into 300 ml tap water for rinsing (1 min). Next, the film was fixed in 300 ml 1:5 fixer (Fisher) and tap water until completely fixed (2-3 min). Finally, the film was air dried for 30 minutes for viewing. After obtaining desired probing with Sculpin NHE-3 DNA probe, the probe was removed by submerging the membrane in 10% SDS and boiling the membrane for 2-3 minutes. Once removal of the primary probe is complete, a secondary Sculpin Actin probe is hybridized to the membrane for detection of actin mRNA as a control using the previous detection procedures.

The second northern blot detection method used $\alpha^{32}\text{P}$ -dCTP radio labeled Sculpin NHE-3 DNA probe for quantification of NHE-3 mRNA expression. The NHE-3 was

radio labeled with $\alpha^{32}\text{P}$ -dCTP following the Prime-a-Gene® (Promega) labeling system protocol (). The Prime-a-Gene® labeling system is based on the method of using random hexadeoxyribonucleotides to prime DNA synthesis in vitro from any linear double-stranded DNA template. With this method it is possible to synthesize probes of extremely high specific activity ($>1 \times 10^9$ cpm/ μg). Since the input DNA is utilized as a template and remains intact during the reaction, minimal amounts of DNA (25 ng) can be labeled to a high specific activity greater than 60%. Sculpin NHE-3 dsDNA template (100 bp) was generated using Faststart taq DNA polymerase PCR kit protocol as described previously. The PCR product concentration was analyzed using spectrophotometry. Next, The PCR was used as a standard concentration using gel electrophoresis for dilution comparison for obtaining an appropriate probe synthesis reaction concentration of (25 ng). The dsDNA band of appropriate concentration was cut out using sterile forceps and razor. The dsDNA was purified from the agarose gel using Gene Clean® spin kit (BIO 101® Systems) according to the manufactures protocol (Appendix). The Sculpin NHE-3 dsDNA was boiled in water bath for 5 minutes and cooled on ice for 10 minutes to denature double stranded DNA. The Sculpin NHE-3 DNA was used as template for the random primer radiolabeling reaction using the (Prime-a-Gene® labeling kit) according to manufactures protocol.

Hybridization procedure was performed using the previously described protocol. Once hybridization was done, the nylon membrane was washed several times for removal of nonspecific probe hybridization. The first step involved two stringency washes of the membrane in 50 ml prewarmed 47°C 2X SSC, 1% (w/v) SDS at 47°C for 30 minutes per wash. Next, the membrane is washed twice more in 50 ml prewarmed 47°C 2X SSC, 0.5

% (w/v) at 47°C for 30 minutes per wash. To determine if probing was a success, the membrane was scanned with a Geiger counter. The isotope reading should be very intense were the probe has hybridized with none or few isotope readings across the remaining membrane area. The bag sealed membrane was taped into place inside a film cassette (Biomax). Next, Biomax film was placed into the cassette according to manufactures instructions in a darkroom. The membrane containing cassette was placed into an -80 C freezer for ~4 hr. depending on initial probe isotope reading. Once exposed, the film was removed from the cassette in the darkroom. While in the darkroom, film is submerged into 300 ml of 1:5 developer (Fisher) and tap water until developed (1-2 min). The developed film was submerged into 300 ml tap water for rinsing (1 min). Next, the film was fixed in 300 ml 1:5 fixer (Fisher) and tap water until completely fixed (2-3 min). Finally, the film was air dried for 30 minutes for viewing. After obtaining desired probing with Sculpin NHE-3 DNA probe, the probe was removed by submerging the membrane in 10% SDS and boiling the membrane for 2-3 minutes. Once removal of the primary probe is complete, a secondary sculpin L8 (Appendix) probe is hybridized to the membrane for detection of L8 RNA as a control using the previous detection procedures.

RESULTS

Isolation of Sculpin Total RNA

Total RNA was isolated from sculpin gill tissue homogenate. Formaldehyde gel electrophoresis showed that rRNA bands (18S and 28S), tRNA band, and mRNA were not degraded or contaminated (Figure 3). The 28S bands were more intense than the 18S bands with both bands being distinct indicating that the total RNA quality was good. Visual interpretation of the gel intensities of the ribosomal bands and spectrophotometer optical density analysis were used to determine a more accurate total RNA concentration for further cDNA synthesis. The optical density was used to quantify the approximate concentration ($\mu\text{g}/\mu\text{l}$) total RNA in the purified sample. The RNA concentration was calculated for the sample in lane 4 (Figure 3) with the brightest intensity. The RNA sample lane 4 was used as a standard to determine an approximate concentration for the other RNA samples base on the band intensity.

RT-PCR using degenerative Sculpin NHE3 primers

The purified extracted sculpin gill total RNA was used as the template for reverse transcription sculpin cDNA synthesis. The sculpin cDNA was used as template for PCR using the sculpin NHE-3 degenerative primer sets (Table 1). The degenerative sculpin primers (F3/R1) PCR cDNA amplification generated a smear of multiple DNA bands with one band at the expected size of ~ 1000 bp (Figure 4) using an annealing temperature of 52°C and 3 minute elongation. The original PCR product was used as template for Nested PCR using internal nested degenerative primer sets (Table 1). The primer sets (F1/RB1) and (F2/RB1) produced two PCR products using a 52°C annealing

temperature (1 minute elongation). Gel electrophoresis shows bands of expected size of ~1000 bp (Figure 5).

Restriction Digest Clone Analysis

A restriction digest of the clones was performed for verification of successful insertion of (F1/RB1) and (F2/RB1) PCR products into bacterial plasmid. The restriction digest indicated the appropriate size PCR product of ~ 1,000 bp (Figure 6) suggesting the correct PCR products of interest were cloned. Sculpin NHE-3 amino acid sequence alignment with Dace NHE-3 (AB055466) shows 73% identity (Figure 7). The generated sculpin NHE-3 cDNA sequence was ~ 1,013 bp. obtained from sculpin gill total RNA.

RACE PCR

Long-horned sculpin gill total RNA was used in 3' and 5' RACE touchdown PCR reactions to obtain the full-length message of the Sculpin NHE-3 homolog. Multiple single band 3' and 5' RACE touchdown products were detected using the primer combinations (SculpNHE3F-nested/GeneRacerTM3' nested primer) for the 3' reaction and (ScpNHE3-176U24/GeneRacerTM5' primer) for the 5' reaction. The 3' RACE touchdown PCR use of the GeneRacerTM3' primer in the initial 3' reaction was not performed due to aberrant nonspecific binding of the GeneRacerTM3' Primer to Sculpin cDNA without a second primer pair. To check for primer contamination causing this phenomenon, a negative control reaction was performed with sterile reagents. The negative control was clearly suggesting no contamination was involved. The most distinct RACE products were detected using an annealing temperature gradient ranging from 50°C to 68°C during the touchdown PCR experiment. The detected RACE PCR products were a high intensity band at the ~1,900 bp DNA marker and a lower intensity band at the ~1400 bp

DNA marker for the 5' reactions (Figure 9) and lower intensity bands at the ~2,100 bp DNA marker for the 3' reactions (Figure 10). Since the touchdown 3' PCR reaction bands were detected with low intensity, a semi-nested 3' PCR reaction was performed using the original touchdown 3' PCR products as template and primer pairs (ScpNHE3-990U21/GeneRacer™3' nested primer) in order to amplify the intensity of the 3' end PCR product. Semi-nested 3' PCR detected a higher intensity band at ~1,400 bp location of the DNA marker (Figure 11). Both the high intensity ~1,900 bp 5' reaction and the ~1,400 bp semi-nested 3' reaction products were gel purified and cloned into pCR®4-TOPO plasmid vector. Transformed bacterial colonies were screened for the appropriate size DNA plasmid inserts using the original primers used to detect the PCR products. PCR screening confirmed the ligation of the ~1,900 bp 5' and the ~1,400 bp semi-nested 3' PCR products insertion into the pCR®4-TOPO plasmid vector (Figure 12). The bacterial plasmid analogous to the PCR screen samples containing the appropriate size bands were purified and sequenced. Automated sequencing of the PCR products was done at Mount Desert Island Biological Laboratory (MDIBL). Sequence results were entered in NCBI BLAST search analysis showing both 5' and 3' RACE products matched other known NHE-3. Both 5'/3' RACE sequences showed homology with dace NHE-3. The sequences was aligned with the original sculpin NHE-3 fragment to determine the full sculpin NHE-3 mRNA transcript using Mac Vector.

The full-length sculpin NHE-3 cDNA contains a 3,097 (Figure 13) nucleotide open reading frame that codes for a protein of 936 amino acids (Figures 14). The consensus sequence data was analyzed using NCBI NIH Blast tool server for comparison to other known sequences. Blast results indicated that at the nucleotide and amino acid

level the sculpin NHE-3 match best with dace NHE-3 out of the database of all complete cDNA clones. Amino acid sequences of all published fish NHE-3 cDNA clones and extrapolated sequence transcript (EST) NHE-3 from complete fish genomes were compared (Figure 15). The 5' region of the sculpin NHE-3 is highly homologous to other fish NHE-3 especially in the conserved transmembrane region. Also, the 5' region of the sculpin NHE-3 revealed a 27 bp (GACACGGGACACGAGACGGGCACTGCA) repeat region (291-452 bp) various lengths (Figure 13) in different sculpin (n=4) that translates to protein (Figure 14). The sculpin NHE-3 (27 bp) repeat caused a gap in the sequence comparison between other fish NHE-3s where the repeat is not present (Figure 15). The 3' region of the sculpin NHE-3 is less homologous with the other fish NHE-3s due to this region is the cytoplasmic region which interacts with substrate in the cytosol (Figure 15). Also, the 3' region of the sculpin NHE-3 (Figure 13) displays a trinucleotide repeat (CCG) of various lengths amongst different sculpin (n=3). The sculpin NHE-3 trinucleotide causes a gap in sequence homology comparison between sculpin NHE-3 and the other fish NHE-3s that do not display this phenomenon (Figure 15). Phylogenetic analysis of the sculpin NHE-3 with other fish NHE-3s shows that the sculpin is most related to the extrapolated stickleback NHE-3, which is another marine teleost.

Hydropathy analysis showed that the sculpin NHE-3 has a similar theoretical membrane topology as the dace NHE-3 with multiple membrane spanning domains in the N-terminal half of the protein and the cytoplasmic domain in the C-terminal half (Figures 16-17). The hydropathy comparison between sculpin NHE-3 and dace NHE3 show the most similarity in the conserved transmembrane spanning domain, whereas; the

hydropathy analysis for the highly variable cytoplasmic domain shows the least similarity. Using new primers (Table 1), RT-PCR was performed to produce fragments of the sculpin NHE-3 sequence. All positive RT-PCR products were ligated into TOPO vectors and transformed into TOPO 10'F cells. Colonies were PCR screened for the correct fragment insert. Colonies containing an insert of the correct size were sequenced, glycerol stocked and stored at -80°C.

Northern Blot Analysis

To determine the expression levels of NHE-3 gill tissue, sculpin were injected with 0.1N (5 ml; 2 mmol kg⁻¹) HCl or water inducing acidosis in the sculpin. Sculpin NHE-3 expression was observed within 0.5 hr., 2 hr. and 4 hr. time intervals. Northern blot examined changes in sculpin NHE-3 mRNA between control and acidotic fish (Figure 20). As a control, northern blot analysis of sculpin L8 RNA expression was examined. The L8 RNA transcribes a highly conserved ribosomal subunit that is constantly expressed for protein translation. Since the amount of L8 subunit does not change, the sculpin L8 RNA expression was compared to the expression of sculpin NHE-3 mRNA.

Kodak imaging system was used to measure the particle density in each Northern blot band (Figure 21-23). In order to accurately calculate the level of NHE-3 expression levels, the individual ratio of NHE-3 to L8 particle density in each sculpin was determined (Table 2). The average NHE-3/L8 particle density ratio of control sculpin were compared to the average ratio of acid injected sculpin in the 0.5 hr., 2 hr., and 4 hr. groups. The particle density measurements of the northern blot bands allowed a quantitative analysis of sculpin NHE-3 expression during acidosis.

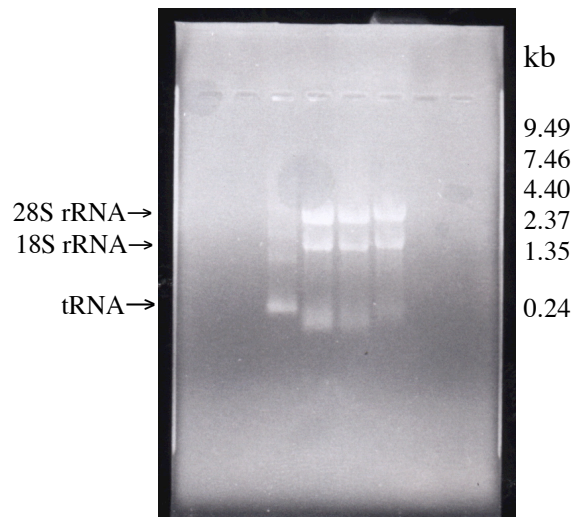


Figure 8: Ethidium bromide stained 1% agarose formaldehyde RNA denaturing gel containing sculpin total RNA (n=3). Lane 3 contains 3.0 µg RNA ladder. Lanes 4, 5, and 6 each contain 5 µg of sculpin total RNA.

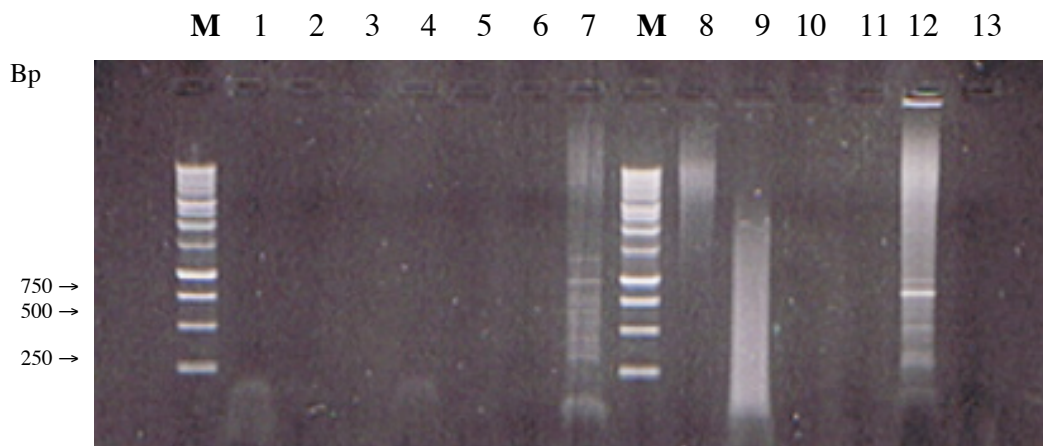


Figure 9: 1% agarose gel stained with EtBr. Lanes labeled M represent a 1 kb DNA ladder. Lane 7 contains multiple bands with one band of expected size at ~ 1,000 bp using primer pair (F3/R1). Lane 12 shows positive control PCR product containing band of appropriate size at ~ 900 bp using sculpin NHE-2 primer pair (Sculp NHE2F/Sculp NHE2R). Lane 13 is the negative control sample with no DNA template that indicates there was no contamination during PCR DNA amplification.

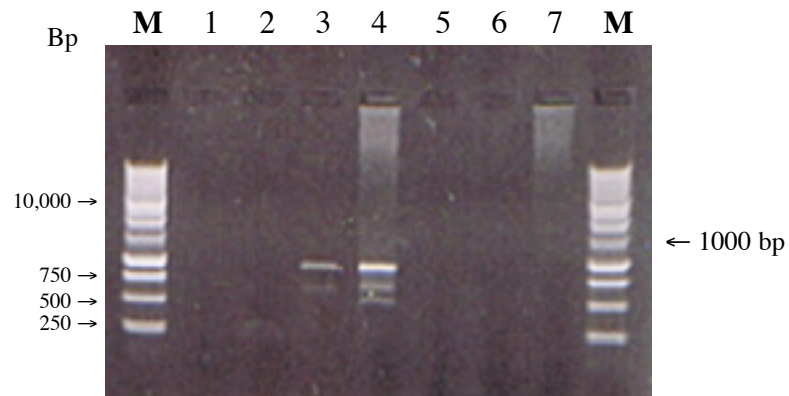


Figure 10: 1% agarose gel stained with EtBr containing putative Na^+/H^+ exchanger-3 (NHE-3) isoform with sculpin (F1/RB1) and (F2/RB1) PCR products. Lane 3 showed the product using F1/RB1 primers of ~1000 bp. Lane 4 showed the product using F2/RB1 primers of ~1000 bp. The smear is an indication of nonspecific binding.

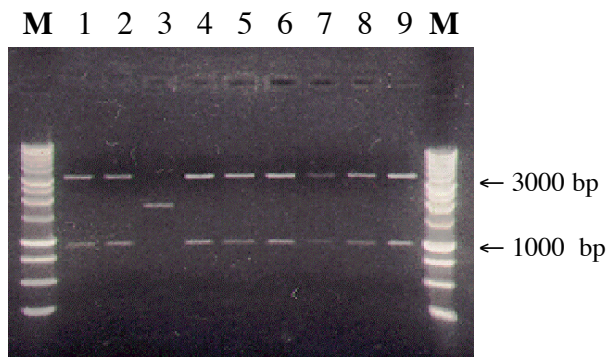


Figure 11: 1% agarose gel stained with EtBr containing nine different restriction digest products cloned plasmid DNA containing sculpin (F1/RB1) and (F2/RB1) PCR primer product inserts.

```

Sculpin: 13   TCTGGGTTTGGAAACACGGGCTTCATCCTCCTCACGCTCCTCTTCATCTTTGTGTACAGAA 72
          |||
Dace: 1269 TCTGGGTTTGGAAACACTGGCTTCATCCTCCTTACAGTCCTCTTCATCATTTGTATTCAGGT 1328
          |||

Sculpin: 73   TTATCGGTGTCTTTTTCCTCACCTGGATCTTGAACAAGTTCAGGCTGGTCCCCGTGGAGT 132
          |||
Dace: 1329 TCATGGGTGTTTCTTCCTCAACTGGATCTTAAATCAGACTCGACTGATTCCTATTGACA 1388
          |||

Sculpin: 133  TTATAGATCAGGTGATTATGAGCTACGGGGGCCTGCGCGGGGCTGTCGCATACGGCCTGG 192
          |||
Dace: 1389 TCACTGACCAGATTGTAATGAGCTACGGTGGTCTTCGAGGAGCAGTGGCATATGGACTCG 1448
          |||

Sculpin: 193  CTGCGATGCTGGACGGGAGCAAGATAAAGGAGAAGAACCTGATGATCTGCACTACTCTCA 252
          |||
Dace: 1449 CAGCTACTTTGGATGAGGCTAAGATCCCAGAGAAGAATTTGATGCTTGGTACAACGCTCA 1508
          |||

Sculpin: 253  TCGTCGTGTACTTCACTGTCATTCTCCAGGGAATGACGATGAAACC 298
          |||
Dace: 1509 TTGTGGTGTATTTACAGTCGTTCTTCAGGGAATCACCATGAAACC 1554
          |||

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Figure 12: Partial sculpin NHE-3 cDNA sequence alignment with Dace NHE-3 (AB055466). The partial Sculpin NHE-3 cDNA showed 73% identity with Dace NHE-3.

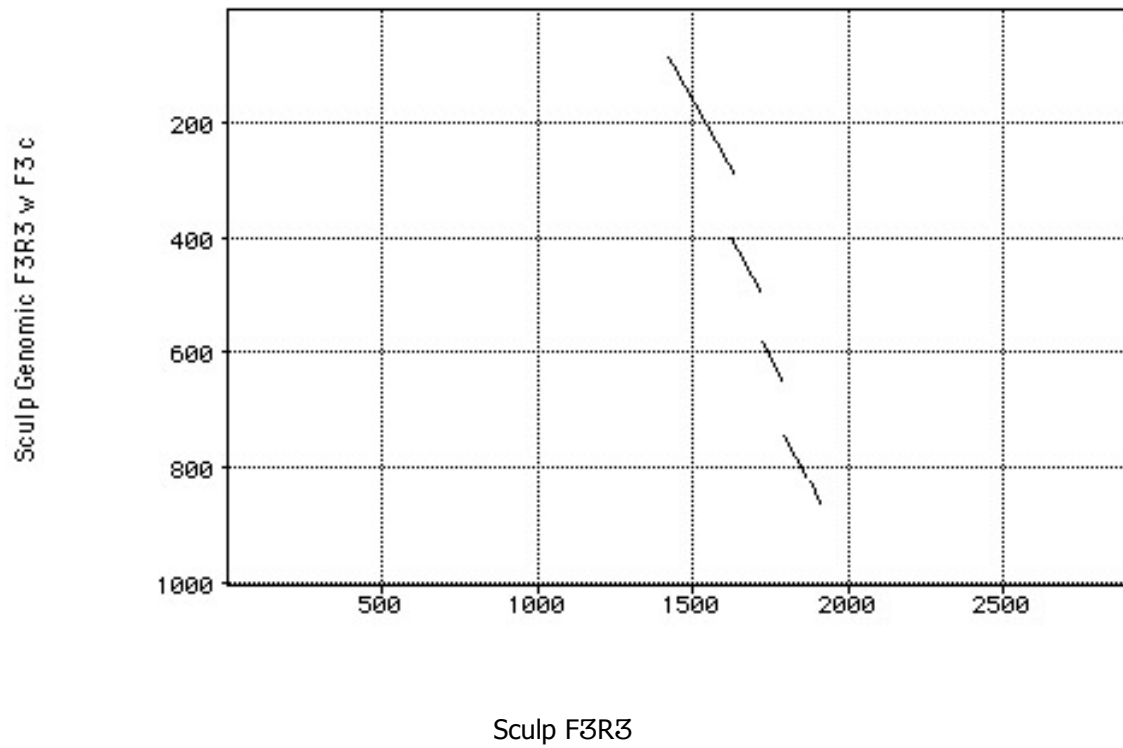


Figure 13: Partial genomic sequence analysis using sculpin primers (F3/R3)w F3c. The genomic sequence was aligned with the partial (F3/R3) sculpin NHE-3 cDNA. The diagonal lines are exons and the spaces between the diagonal lines are introns.

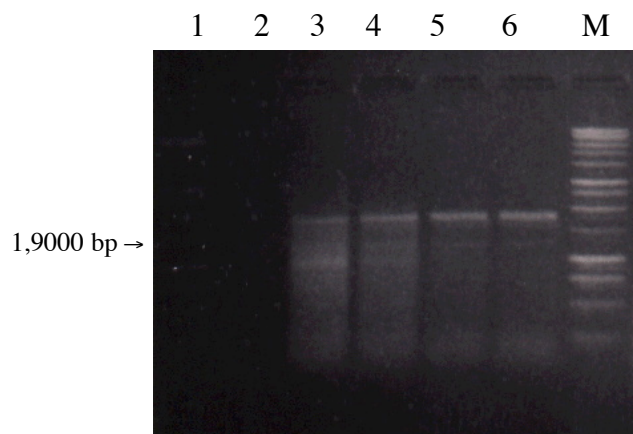


Figure 14: Ethidium bromide stained 1% agarose gel of 5'-RACE products. PCR product synthesis in lane 1 negative control is absent indicating there was no contamination. Lanes 2-5 are 5' RACE products at annealing temperatures ranging from 50°C to 68°C. Lane 5 PCR produced an appropriate distinct band of approximately ~1,900 bp. at 68°C annealing temperature.

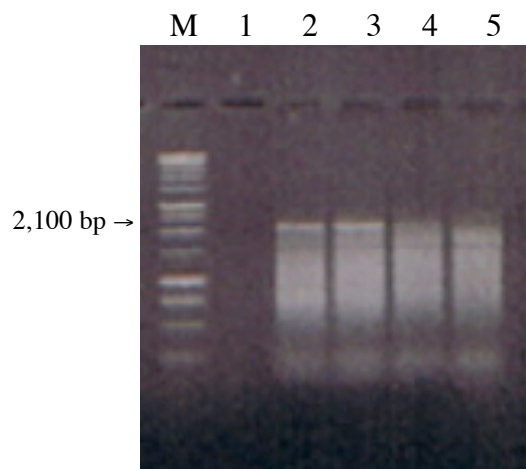


Figure 15: Ethidium bromide stained 1% agarose gel of 3'-RACE products. PCR product synthesis in lane 1 negative control is absent indicating there was no contamination. Lanes 2-5 are 3' RACE products at annealing temperatures ranging from 50°C to 68°C. Lanes 2 and 3 PCR produced an appropriate band of approximately ~2,100 bp. at 50°C and 55°C annealing temperatures.

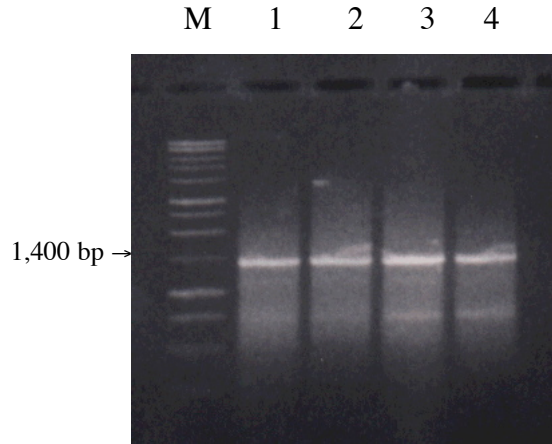


Figure 16: Ethidium bromide stained 1% agarose gel of 3' Nested RACE PCR. The PCR product synthesis in lane 5 negative control is absent indicating there was no contamination. Lanes 1-4 are 3' Nested RACE products at annealing temperatures ranging from 50°C to 68°C. Lanes 1-4 PCR produced an appropriate band of approximately ~1,400 bp. at 50°C to 68°C annealing temperatures.

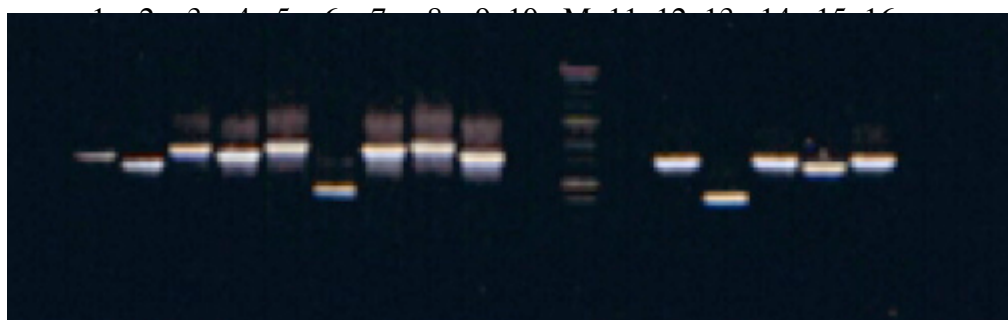


Figure 17: PCR analysis of RACE PCR cDNA plasmid clones show amplification of an expected size of 1,400 kb for 3' RACE PCR product insertion in lanes (12, 14, 16) and for 1,900 kb for 5' RACE PCR product insertion in lanes (5, 7, 8). Negative controls in lanes 10 and 11 are free of DNA bands indicating there was no contamination in the reaction.


```

      10      20      30      40      50      60      70
MAATRRFALFLCMLLMVSRGPSLASEDTGHETGTVDTGHETGTA DTGHETGTA DTGHETGTA DTGHETGT
      80      90     100     110     120     130     140
ADTGHETGTA DTGHETGTA DTGDHQGTTTDSHDSGNGDTHDESTGEGGGHGGGPITTLPIVSWKWEHVS
      150     160     170     180     190     200     210
TPYLVALWILVSWLCKLVIEANHHVTSVIPESALLICFGFLGGI IWGADMVQTFKLTPTLFFFYLLPQI
      220     230     240     250     260     270     280
ILDAGYSMPNKLFFSNMGAILIYAVIGTCWNAASLGLSLWGCHQGGAMGDLDIGLLQYLLFGSLIAAVDP
      290     300     310     320     330     340     350
VAVIAVFEQVHVNEVLFILVFGESLLNDGVTTVLNFVDFAVSLGGSGIDAVEIIKGIISFFVVAFGGSL
      360     370     380     390     400     410     420
VGFLFGLLISFLTRGTKNIQIIEPAFVFLGYLAYLTAEMLSLSAILSIVFCGMCCQKYINANMDERSVA
      430     440     450     460     470     480     490
TVRYVMKVLANGSETIIFVFLGISAIDKTIWVWNTGFILLTLLFIFVYRIIGVFFLTWILNKFRLVPVEF
      500     510     520     530     540     550     560
IDQVIMSYGGLRGAVAYGLAAML DGSKIKEKNLMICTTLIVVYFTVILQGMTMKPLVNWLVKKASPTL
      570     580     590     600     610     620     630
TLIEKLQNKVFDHMLVAIEDVSGQIGHNYMRDKWNHFEEKWMSRILMKPSARKNRDVFNVFHQLNMKDA
      640     650     660     670     680     690     700
MSYVAEGERRGSLEFVRNDTAFIDFRKKFGDDCEVM PDITADTSDDHCGSSTSGRDPVPSV SLEMHEQN
      710     720     730     740     750     760     770
MKGVRETEDINTHLLQQHLYKGRKQHRHRYRSRSHFNVNTDDGEVQEIFQRTMRNRLESFKSAKMGVAPQ
      780     790     800     810     820     830     840
KKIAKHTKKDQQQKMPNGKSMDKSKSYSGDEDFEFSEGDNASGLEASGGSYPFPMV TYRAGAGIENPAF
      850     860     870     880     890     900     910
MPDLDPMAPAQIP PWLADMELDCGPVAPSQRAQLRLPWT PSDLRRLAPLRISTRSTDSFAQ AAAAAAAAAA
      920     930
PAPPQTANKPPPPPPPPRPPSSHM*

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Figure 19: Full-length amino acid sequence for Sculpin NHE-3. Complete sequence is 934 amino acids (aa) in length for this clone. The 27 bp repeat is translated to (DTGHETGTA) 1-7 region (27-89 aa) is bold (red) and alternating boxes. The trinucleotide repeat is translated to (AAAAAAAAA) 1-10 region (902-912 aa) is bold (purple) and with a box.

		130	140	150
<i>Sculpin</i>	T	G E G - - - G	G H G G G P	I T T L P I V S V K V E H V S
<i>Zfishb</i>	S	G N S - - - -	S H S A T	I T T L P I V T V K V H H V E
<i>Dace</i>	N	- - - - -	V T T T T	I T T L P I V T V K V H H V E
<i>Stickleback</i>	T	P N Q S H A D H	G H G S S A	V A G I P V V T F K V H H V E
<i>Puffer</i>	-	G G S - - - -	G H E A A N	I T T L P I V S V K V H H V E
<i>Fugu</i>	-	G Q G - - - -	G H E A E P	I T T L P I V S V K V E H V K
<i>Stingray</i>	H	G G N - - - -	E T S G H Q P G F R	I V S F R V E H V Q
<i>Zfisha</i>	D	P H H - - - -	H Q T E N L	T G L P I V T F K V H H V E
	G	S H A D G H	.	I T T L P I V . W K V H H V E

		160	170	180
<i>Sculpin</i>	T	P Y L V A L W I L V S	V L C K - L V I E A	N H H V T S V I
<i>Zfishb</i>	T	P Y L V A L W I L T C	V L C K L - V T E L	N H N I T S V I
<i>Dace</i>	T	P Y L V A L W I L T C	V L C K L - V C E L	N H S I T S V I
<i>Stickleback</i>	T	P Y Q V T L W I L V A	G L A K T L I I E A	N H H V T N V I
<i>Puffer</i>	A	P Y L V A L W V L V G	V L C K - L V I E A	N H Q V T N V I
<i>Fugu</i>	T	P Y L V A L W V L V G	V L C K - L V I E A	N H Q V T N I I
<i>Stingray</i>	A	P Y I I A V W I L V A	S L G K - I V F H L	S E K V T S V V
<i>Zfisha</i>	T	P Y L V A L W V F V A	G L A K L S V I Q L	N H S I T G V F
	T	P Y L V A L W I L V	W L C K L L V I E	N H V T V I

		190	200	210
<i>Sculpin</i>	P	E S A L L I C F	G F L L G G I I V G A D M V	Q T F K L T P
<i>Zfishb</i>	P	E S G L L I I L	G F I L G G I V V G A D K A	Q T F K L I P
<i>Dace</i>	P	E S G L L I M M	G F I L G G I V V G A D K A	Q T F K L I P
<i>Stickleback</i>	P	E S A L L I C F	G F I L G G I I V G A D K V	Q T F K L N P
<i>Puffer</i>	P	E S A L L I C F	G F I L G G M V V G A D K V	Q S F K L D P
<i>Fugu</i>	P	E S A L L I C F	G F I L G G I V V G A D M V	Q S F K L D P
<i>Stingray</i>	P	E S A L L I V L	G L I L G G I V V A A D H T A	S F T L T P
<i>Zfisha</i>	P	E S G L L I I L	G F I L G G I V V G A D K A	Q T F K L L P
	P	E S A L L I	G F I L G G I V V G A D K	Q T F K L P

		220	230	240
<i>Sculpin</i>	T	L F F F Y L L P Q I I L D A G Y S	M P N K L F F S	N M G A
<i>Zfishb</i>	V	N F F F Y L L P Q I I L D A S Y C	M P N K L F F G	N L G A
<i>Dace</i>	V	N F F F Y L L P Q I V L D A S Y C	M P N K L F F S	N L G A
<i>Stickleback</i>	T	V F F F Y L L P Q I I L D T G Y S	M P N K L F F G	N M G A
<i>Puffer</i>	R	V F F F Y L L P Q I I L D T G Y S	M P N K L F F S	N L G G
<i>Fugu</i>	T	V F F F Y L L P Q I I L D T G Y S	M P N K L F F S	N L G G
<i>Stingray</i>	T	V F F F Y L L P P I V L D A G Y F	M P N R H F F G	N I G S
<i>Zfisha</i>	S	T F F F Y L L P Q I I L D A S Y F	M P N K L F F R	N L G A
	.	F F F Y L L P Q I I L D A G Y	M P N K L F F	N L G A

		250		260		270
<i>Sculpin</i>		I L I Y A V I G T C V N A A S	L	G L S L W G C H	Q	G G A M G
<i>Zfishb</i>		I L V H A I I G T C V N A G T	V	G I A L W A C Y E		G G A M G
<i>Dace</i>		I L V H A I I G T C V N A G T	V	G I A L W A C Y E	W	G A M G
<i>Stickleback</i>		I L V Y A V I G T C V N A A S	L	G L S L W G C H	Q	G G A M G
<i>Puffer</i>		I L V Y A I I G T C V N A A S	L	G L S L W G C H	K	G G A M G
<i>Fugu</i>		I L V Y A I I G T C V N A A S	L	G L S L W G C H	M	G G A M G
<i>Stingray</i>		I L M Y A V I G T I V N A A T	T	G L S L Y G V F L S	G I	M G
<i>Zfisha</i>		I L I Y A I F G T C V N A A A	V	G L S L W G C H	E	A G A M G
		I L V Y A I I G T C V N A A .		G L S L W G C H		G G A M G

		280		290		300
<i>Sculpin</i>		D L D I G L L Q Y L L F G S L I A A V D P V A V I A V F E	Q			
<i>Zfishb</i>		T L N I G C L Q F L L F G A L M S	A V D P V A V I A V F E	E		
<i>Dace</i>		T L N I G P L Q F L L F G S L M S	A V D P V A V I A V F E	E		
<i>Stickleback</i>		D L D I G L L Q Y L L F G S L I A A V D P V A V I A V F E	Q			
<i>Puffer</i>		D L D I G L L Q Y L L F G S L I A A V D P V A V I A V F E	Q			
<i>Fugu</i>		D L D I G L L Q Y L L F G S L I A A V D P V A V I A V F E	Q			
<i>Stingray</i>		D L K A G L L E F L L F G S L I A A V D P V A V L A V F E	E			
<i>Zfisha</i>		D L N I G L L Q F L L F G S L I A A V D P V A V I A V F E	E			
		D L I G L L Q . L L F G S L I A A V D P V A V I A V F E				

		310		320		330
<i>Sculpin</i>		V H V N E V L F I L V F G E S L L N D G V T V V L F N V F D				
<i>Zfishb</i>		V H V N E V L Y I L V F G E S L L N D G V T V V L Y N V F D				
<i>Dace</i>		V H V N E V L Y I L V F G E S L L N D G V T V V L Y N V F D				
<i>Stickleback</i>		V H V N E V L F I L V F G E S L L N D G V T V V L F N V F D				
<i>Puffer</i>		V H V N E V L F I M V F G E S L L N D G V T V V L F N V F D				
<i>Fugu</i>		V H V N E V L F I M V F G E S L L N D G V T V V L F N V F D				
<i>Stingray</i>		V H V N E V L F I I V F G E S L L N D A V T V V L Y N V F D				
<i>Zfisha</i>		V H V N E V L F I L V F G E S L L N D G V T V V L Y N V F D				
		V H V N E V L F I L V F G E S L L N D G V T V V L . N V F D				

		340		350		360
<i>Sculpin</i>		A F V S L G G S G I D A V	E I I K G I I S F F V V A F G G S			
<i>Zfishb</i>		A F V S L G G P K I N A A	E I I K G I V S F A V V A F G G S			
<i>Dace</i>		A F V T L G G P R I N A E	E I I K G I I S F F V V A F G G S			
<i>Stickleback</i>		A F V S L G G A R I N A V	E I I K G I I S F F V V A F G G S			
<i>Puffer</i>		A F V S L G G S K I N A A	E I I K G I V S F F V V A F G G S			
<i>Fugu</i>		A F V S L G G S K I N A V	E I I K G I V S F F V V A F G G S			
<i>Stingray</i>		S F V A V G A G N I Q G L D Y F K G I V S F F V V S L G G T				
<i>Zfisha</i>		G F V S L G G P M I D A A	E I F K G I C S F V V V A F G G S			
		A F V S L G G . I N A	E I I K G I . S F F V V A F G G S			

		490		500		510																								
<i>Sculpin</i>	I	I	G	V	F	F	L	T	W	I	L	N	K	F	R	L	V	P	V	E	F	I	D	Q	V	I	M	S	Y	G
<i>Zfishb</i>	M	M	G	V	F	F	L	N	W	I	L	N	Q	S	R	L	I	P	I	D	L	T	D	Q	L	V	M	G	Y	G
<i>Dace</i>	F	M	G	V	F	F	L	N	W	I	L	N	Q	T	R	L	I	P	I	D	I	T	D	Q	I	V	M	S	Y	G
<i>Stickleback</i>	I	I	G	V	F	F	L	T	W	I	L	N	R	Y	R	L	V	P	V	E	F	I	D	Q	V	I	M	S	Y	G
<i>Puffer</i>	I	I	G	V	F	C	L	T	W	I	L	N	K	Y	R	L	V	P	L	E	F	I	D	Q	V	I	M	S	Y	G
<i>Fugu</i>	I	I	G	V	F	F	L	T	W	I	L	N	K	F	R	L	V	P	L	E	F	I	D	Q	V	I	M	S	Y	G
<i>Stingray</i>	V	I	G	V	V	V	Q	T	W	I	L	N	H	F	R	V	V	Q	L	E	I	I	D	Q	V	V	M	S	Y	G
<i>Zfisha</i>	I	I	G	V	F	M	F	T	W	M	L	N	K	F	R	L	V	P	V	E	L	I	D	Q	V	V	M	S	Y	G
	I	I	G	V	F	F	L	T	W	I	L	N	.	.	R	L	V	P	.	E	.	I	D	Q	V	.	M	S	Y	G

	520																530										540									
<i>Sculpin</i>	G	L	R	G	A	V	A	Y	G	L	A	A	M	L	D	G	S	K	I	K	E	K	N	L	M	I	C	T	T	L						
<i>Zfishb</i>	G	L	R	G	A	V	A	Y	G	L	A	A	S	L	D	E	N	K	I	P	E	K	N	L	M	L	G	T	T	L						
<i>Dace</i>	G	L	R	G	A	V	A	Y	G	L	A	A	T	L	D	E	A	K	I	P	E	K	N	L	M	L	G	T	T	L						
<i>Stickleback</i>	G	L	R	G	A	V	A	Y	G	L	A	T	L	L	N	E	S	K	I	K	E	K	N	L	M	I	C	T	T	L						
<i>Puffer</i>	G	L	R	G	A	V	A	Y	G	L	A	T	M	L	D	E	T	R	I	K	E	K	N	L	M	I	C	T	T	L						
<i>Fugu</i>	G	L	R	G	A	V	A	Y	G	L	A	T	L	L	D	E	N	K	I	K	E	K	N	L	M	I	C	T	T	L						
<i>Stingray</i>	G	L	R	G	A	V	A	F	A	L	V	A	L	L	N	E	N	N	V	Q	E	K	S	L	F	V	S	T	T	I						
<i>Zfisha</i>	G	L	R	G	A	V	A	Y	G	L	A	A	M	L	D	E	S	R	I	P	E	K	K	L	M	I	S	T	T	L						
	G	L	R	G	A	V	A	Y	G	L	A	A	.	L	D	E	.	K	I	.	E	K	N	L	M	I	.	T	T	L						

		550		560		570																								
<i>Sculpin</i>	I	V	V	Y	F	T	V	I	L	Q	G	M	T	M	K	P	L	V	N	W	L	K	V	K	K	A	S	P	T	E
<i>Zfishb</i>	I	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	N	W	L	K	V	K	K	A	T	Q	S	D
<i>Dace</i>	I	V	V	Y	F	T	V	V	L	Q	G	I	T	M	K	P	F	V	Q	W	L	K	V	K	K	A	T	I	S	D
<i>Stickleback</i>	L	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	N	W	L	K	V	K	K	A	A	V	S	E
<i>Puffer</i>	I	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	T	W	L	K	V	R	R	A	A	S	G	D
<i>Fugu</i>	I	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	T	W	L	K	V	R	R	A	A	A	G	D
<i>Stingray</i>	I	V	V	Y	F	T	V	I	L	Q	G	L	T	I	K	P	L	V	K	W	L	K	V	K	R	S	E	Q	K	D
<i>Zfisha</i>	I	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	Q	W	L	K	V	K	R	A	A	H	S	D
	I	V	V	Y	F	T	V	I	L	Q	G	I	T	M	K	P	L	V	.	W	L	K	V	K	.	A	.	D	.	.

	580															590															600														
<i>Sculpin</i>	L	T	L	I	E	K	L	Q	N	K	V	F	D	H	M	L	V	A	I	E	D	V	S	G	Q	I	G	H	N	Y															
<i>Zfishb</i>	L	T	L	N	G	K	L	N	N	R	V	F	E	H	T	L	T	G	M	E	D	I	C	G	R	M	G	D	N	W															
<i>Dace</i>	L	T	L	N	G	K	I	N	N	R	T	F	E	H	T	L	T	A	M	E	D	I	C	G	R	M	G	D	N	W															
<i>Stickleback</i>	L	T	L	I	E	K	L	Q	S	K	V	F	D	H	M	L	V	A	I	E	D	I	S	G	Q	I	G	H	N	Y															
<i>Puffer</i>	V	S	L	M	E	K	V	H	R	K	M	F	D	H	I	L	V	A	I	E	D	I	S	G	Q	I	G	H	N	Y															
<i>Fugu</i>	V	T	L	M	E	K	V	H	N	K	M	F	D	H	I	L	V	A	I	E	D	V	S	G	Q	I	G	H	N	Y															
<i>Stingray</i>	P	L	L	N	E	K	L	H	G	R	A	F	D	H	I	L	S	A	I	E	D	I	S	G	Q	I	G	H	N	Y															
<i>Zfisha</i>	L	K	L	V	E	K	L	N	N	R	A	F	D	H	I	L	A	A	V	E	D	I	C	G	R	I	G	D	N	W															
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<i>Zfishb</i>	F	D	I	D	S	V	P	V	T	S	V	M	K	N	P	I	P	S	V	S	L	D	I	H	E	Q	E	R	R	G		
<i>Dace</i>	F	G	I	D	N	V	P	V	N	S	F	M	K	N	Q	I	P	S	V	S	L	D	I	H	E	Q	D	R	K	S		
<i>Stickleback</i>	S	A	M	S	-	-	-	S	M	S	R	I	D	Q	V	P	S	V	S	L	E	M	H	E	Q	N	M	K	G			
<i>Puffer</i>	E	P	-	-	-	-	-	T	A	V	G	R	D	P	V	P	S	V	C	L	D	I	D	E	Q	A	M	K	A			
<i>Fugu</i>	G	P	-	-	-	-	-	T	G	F	R	R	D	P	V	P	S	V	C	L	E	M	D	E	P	A	M	K	G			
<i>Stingray</i>	Q	S	I	V	D	S	S	V	S	A	V	L	R	E	S	T	S	E	V	C	L	D	M	H	A	L	E	N	R	V		
<i>Zfisha</i>	F	G	I	E	N	I	S	V	A	T	I	V	K	D	T	I	P	S	V	S	L	D	I	H	G	Q	D	G	K	N		
		I		V	.			.	D	.	P	S	V	S	L	D	H	E	Q		K											

								730										740														750
<i>Sculpin</i>	V	R	E	T	E	-	-	D	I	N	T	H	H	L	L	Q	Q	H	L	Y	K	G	R	K	Q	H	R	H	R	Y		
<i>Zfishb</i>	M	S	N	-	-	-	-	D	L	N	A	H	H	L	L	E	Q	H	L	Y	K	S	R	R	N	N	R	A	T	Y		
<i>Dace</i>	M	P	D	-	-	-	-	D	L	S	A	H	H	L	L	E	Q	H	L	Y	K	S	R	R	Y	N	R	P	T	Y		
<i>Stickleback</i>	V	R	E	T	E	-	-	D	I	N	T	H	H	L	L	Q	Q	H	L	Y	K	G	R	K	Q	H	R	H	R	Y		
<i>Puffer</i>	T	R	E	S	E	-	-	D	I	N	T	H	H	L	L	Q	Q	H	L	Y	K	S	R	K	N	H	R	H	R	Y		
<i>Fugu</i>	T	R	E	S	E	-	-	D	I	N	S	H	H	L	L	Q	Q	H	L	Y	K	G	R	K	H	H	R	H	R	Y		
<i>Stingray</i>	K	S	Q	K	H	R	E	E	S	V	T	H	R	M	L	Q	Q	H	L	Y	K	P	R	Q	R	Y	R	L	N	Y		
<i>Zfisha</i>	V	V	D	-	-	-	-	D	I	N	A	H	R	L	L	Q	Q	H	L	Y	R	S	R	K	H	H	H	H	K	Y		
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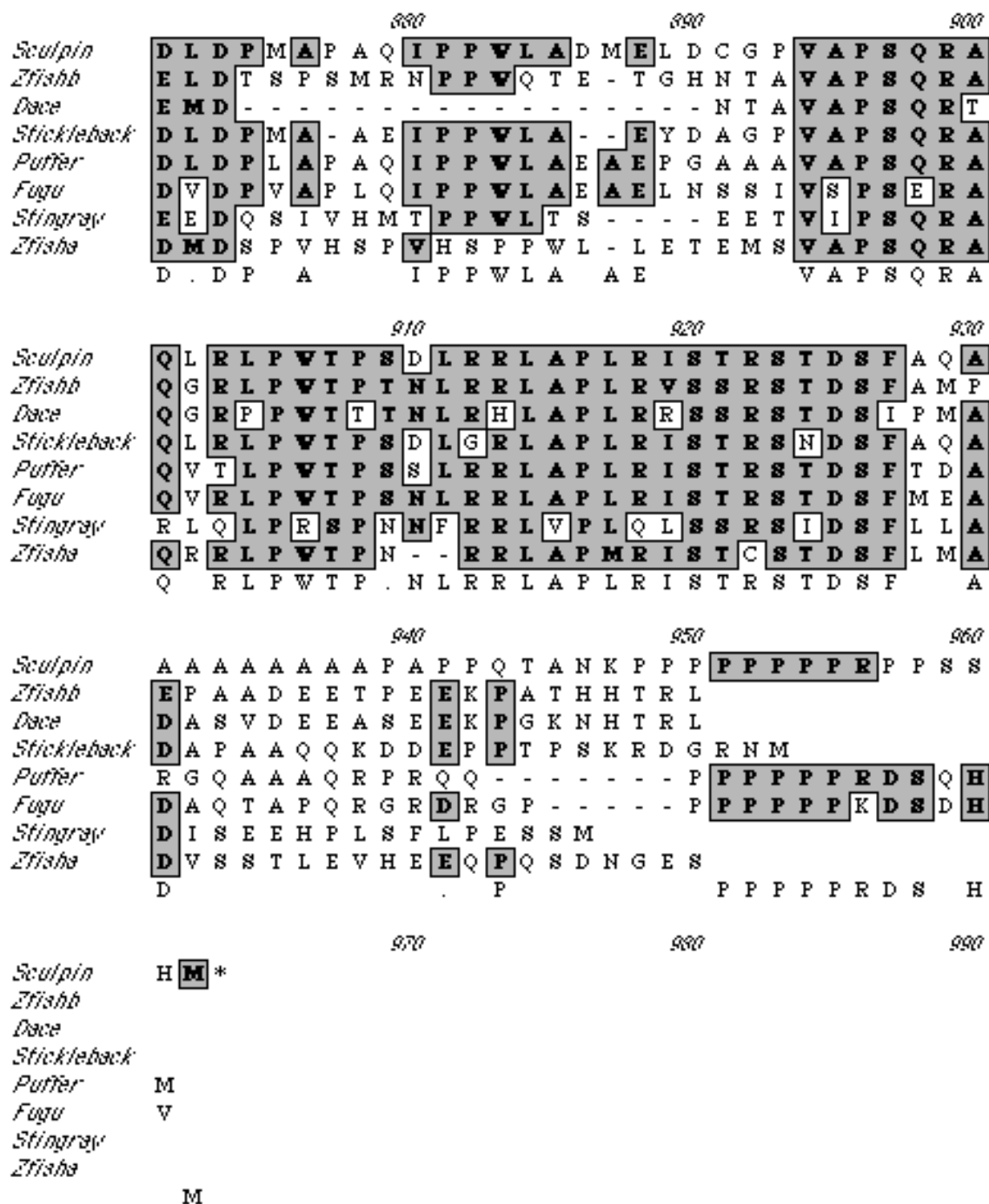


Figure 20: ClustalW alignment of all the complete fish NHE-3 sequences with the sculpin NHE-3 sequence. Homologous regions are shaded. Sequences for each of the published NHE-3 sequences can be obtained from the BLAST NIH webserver.

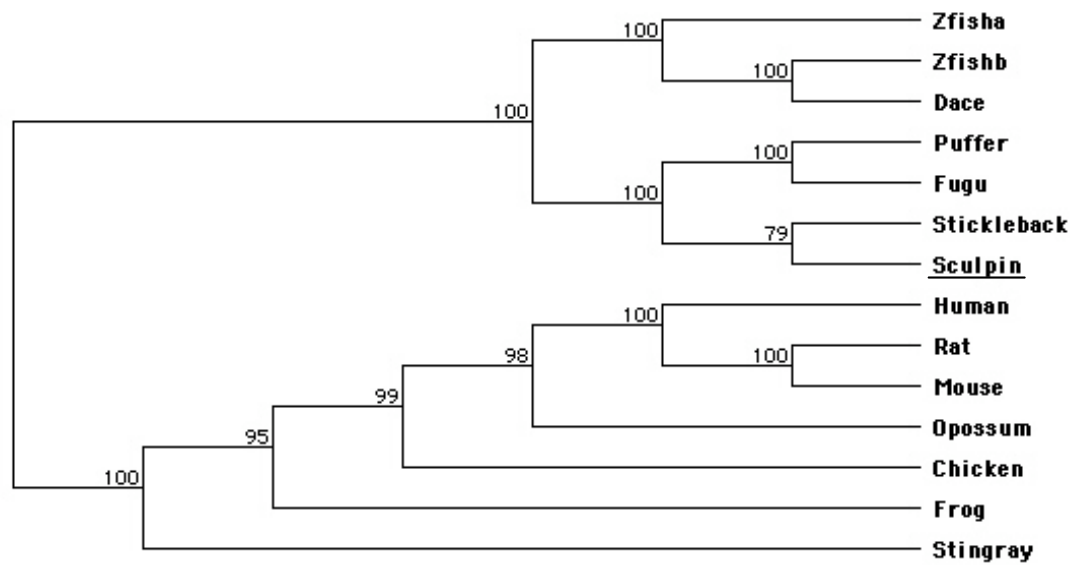


Figure 21: Phylogenetic tree of the fish NHE-3 gene family with the sculpin NHE-3 sequence. The tree shows that the sculpin NHE-3 (under lined) is located closest to the NHE-3 of the marine teleost stickleback. Numbers represent the differences between two sequences.

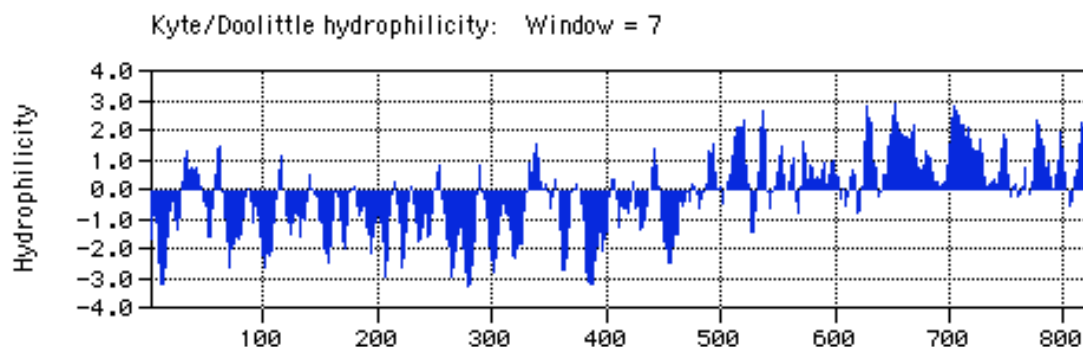


Figure 22: Hydrophilicity plot for the dace NHE-3 protein was determined according to the algorithm of Kyte and Doolittle using a window of 7 amino acids. Positive values correspond to hydrophilic regions and negative numbers correspond to hydrophobic segments.

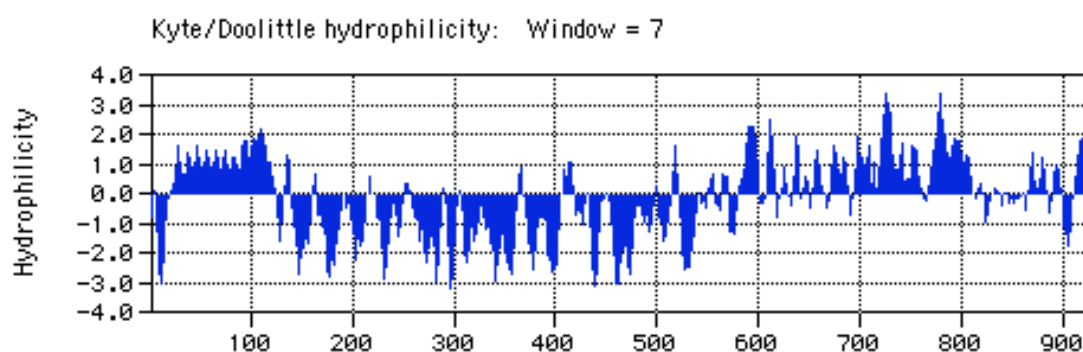


Figure 23: Hydrophilicity plot for the sculpin NHE-3 protein was determined according to the algorithm of Kyte and Doolittle using a window of 7 amino acids. Positive values correspond to hydrophilic regions and negative numbers correspond to hydrophobic segments.

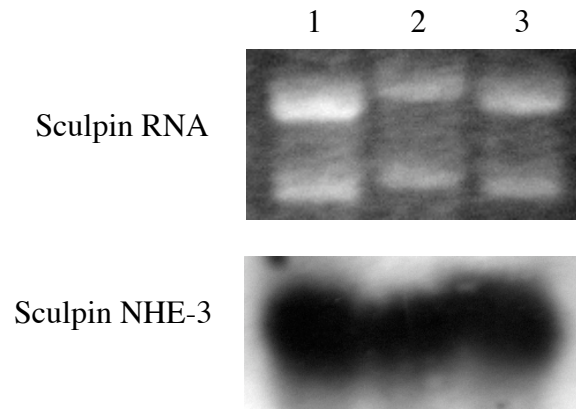


Figure 24: Chemiluminescent northern blot hybridization of sculpin gill total RNA (n=3) show a band of approximately 3.8 kb using a digoxegenin labeled sculpin NHE-3 DNA probe in lanes 1-3.

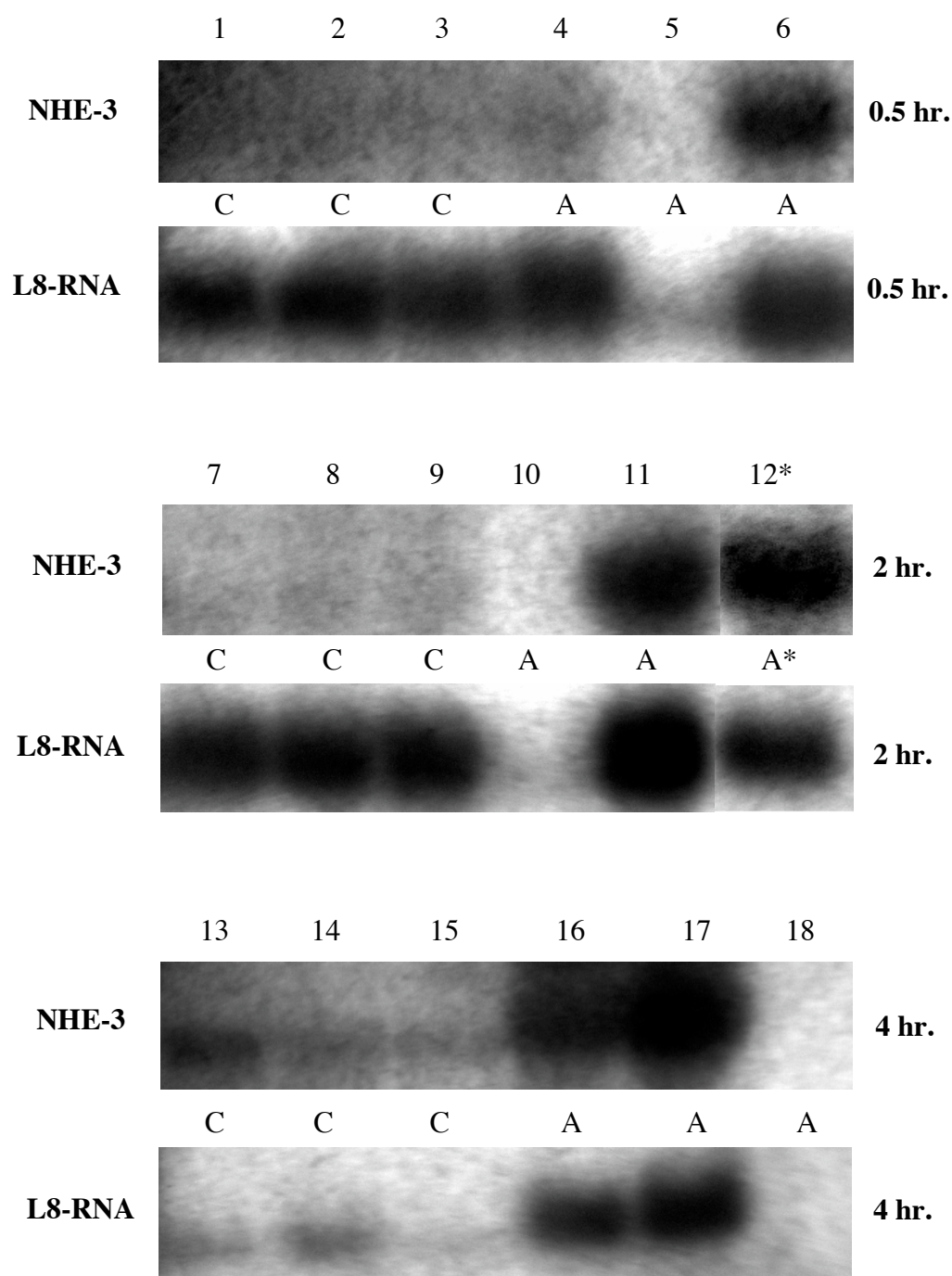


Figure 25: Northern blot of sculpin gill total RNA hybridized with $\alpha^{32}\text{P}$ radio labeled probes for NHE-3 (top) and ribosomal L8 (bottom). Control (C) and acid (A) loaded fish are shown (N=3 each group). Longer exposure times revealed the NHE-3 transcript was also present (but at lower concentrations) in the control fish (not shown).

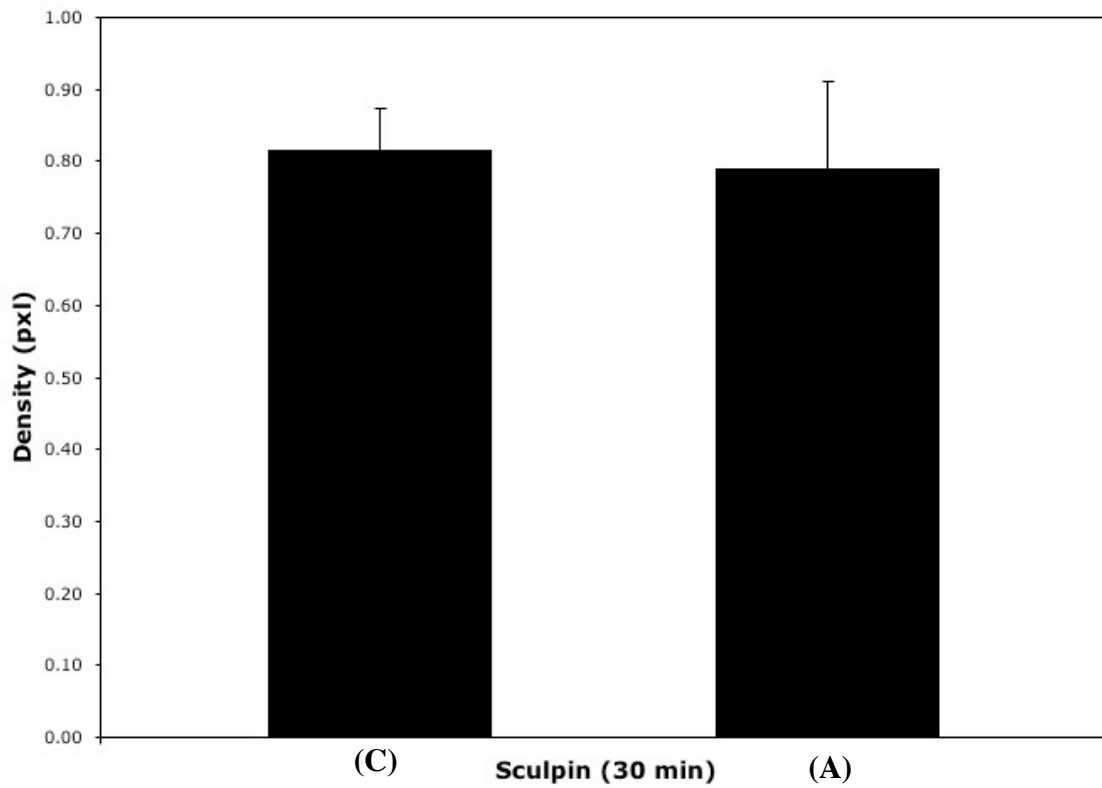


Figure 26: Average scanning densitometry of Northern Blot analysis of NHE-3 mRNA in gills of normal control (C) and acidotic (A) sculpin 30 minutes post-infusion. Results are graphed as mean pixel densities (bars represent SE). The ratio of density change between groups was determined and statistical analysis (unpaired t-test) of the mean NHE-3 for each group revealed no significant differences (n=3 for each group).

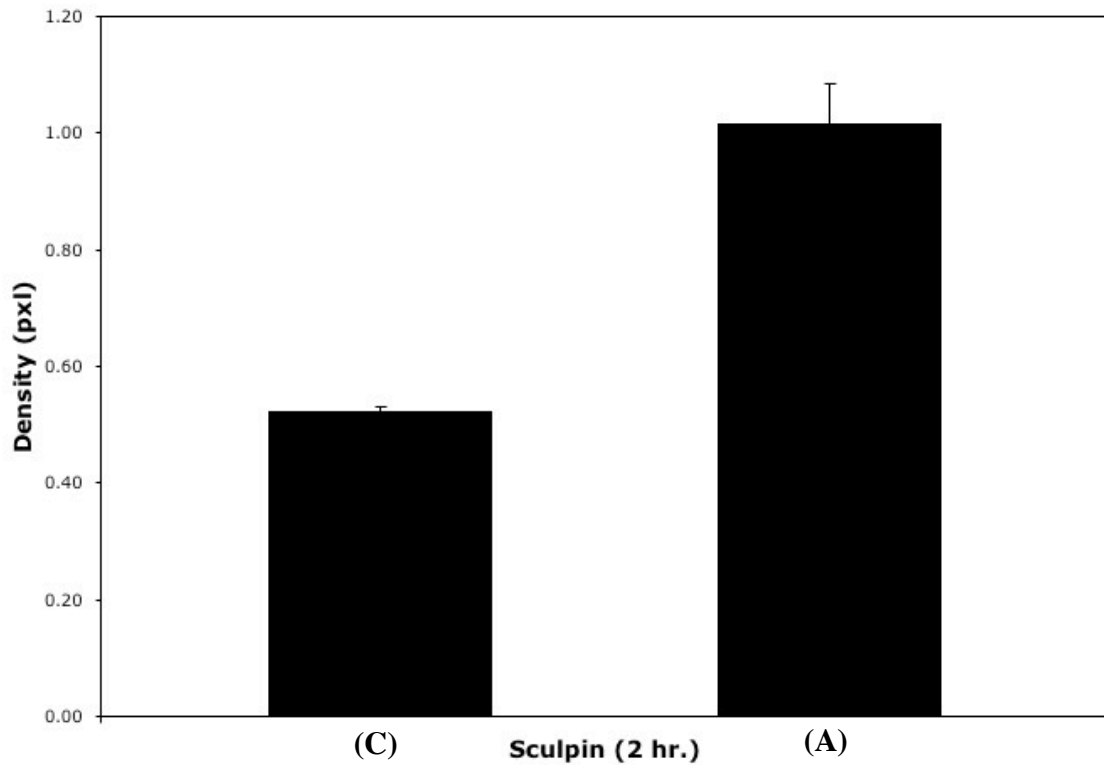


Figure 27: Average scanning densitometry of Northern Blot analysis of NHE-3 mRNA in gills of normal control (C) and acidotic (A) sculpin two hours post infusion. Results are graphed as mean pixel densities (bars represent SE). The ratio of density change between groups was determined and statistical analysis (unpaired t-test) of the mean change in NHE-3 indicated a significant difference ($p < 0.02$; $n=3$ control, $n=2$ acid group).

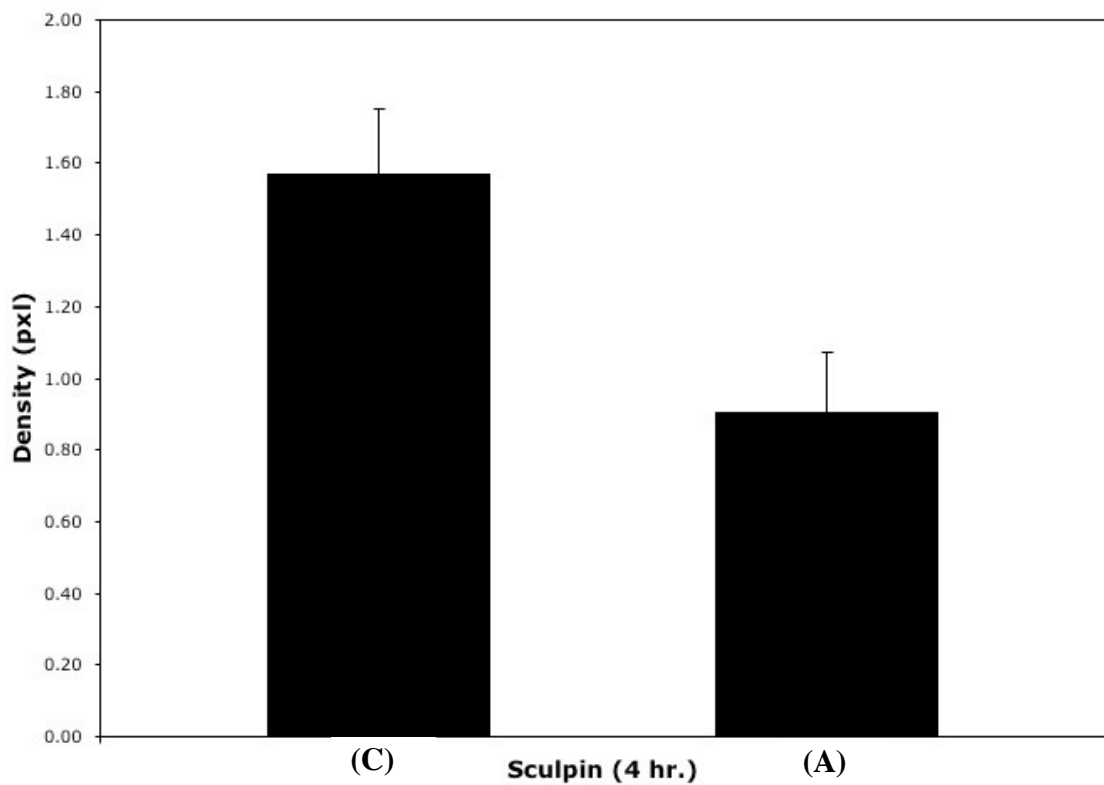


Figure 28: Average scanning densitometry of Northern Blot analysis of NHE-3 mRNA in gills of normal control (C) and acidotic (A) sculpin four hours post-infusion. Results are graphed as mean pixel densities (bars represent SE). The ratio of density change between groups was determined and statistical analysis (unpaired t-test) of the mean NHE-3 for each group revealed no significant differences (n=3 for each group).

DISCUSSION

To date there has been no characterization of the NHE-3 isoform in marine teleost fish gill epithelia. NHE-3 is the most highly expressed isoform in mammalian kidney proximal tubules and is the primary isoform for Na^+/Cl^- reabsorption and H^+ excretion in the tubules of mammals. Characterization of NHE-3 in marine fish will allow novel insight into the role of NHE-3 in acid-base and ion regulation across the gills. Further expression studies will allow an understanding of the interactions between the different NHE isoforms.

Sculpin degenerate primers (F1/RB1) and (F2/RB2) produced a partial NHE-3 cDNA homologous (73%) to Dace NHE-3 cDNA sequence (Figure 7). The remaining 3' and 5' sequence of the sculpin NHE-3 was obtained using gene specific primers by RACE PCR (Figure 12). The full-length sculpin NHE-3 cDNA contains a 3,057 bp nucleotide open reading frame, which translates to a protein of 934 amino acids for this clone (Figure 13-14). To confirm the presence of NHE-3 mRNA in the sculpin gill, chemiluminescent northern blot using digoxigenin labeled sculpin NHE-3 DNA probe detected a 3.8 kb mRNA band (Figure 19). A sculpin sequence alignment and phylogenetic tree comparison to all other known fish NHE-3s shows the highest homology to the genome sequence derived stickleback (teleost) NHE-3 (Figure 15-16). Hydrophilicity analysis showed that the sculpin NHE-3 has the same membrane topology as the dace NHE-3 and is predicted to have 12 membrane spanning region and a long cytoplasmic C terminal region (Figure 17-18). The transmembrane region of the sculpin NHE-3 is very conserved showing high homology with other fish NHE-3 (Figure 15). However, the c-terminal cytoplasmic tail is highly variable (Figure 15) and may be

due to the specific function of the NHE-3 isoform in the gill epithelia (Bookstein, et. al., 1997).

Cloning of the sculpin NHE-3 isoform revealed nucleotide repeats in the mRNA coding region upstream and downstream of the conserved transmembrane domain. The upstream 5' N-terminal region repeat consisted of variable number tandem-repeats (VNTRs) with 27 bp (GACACGGGACACGAGACGGGCACTGCA) repeat unit (Figure 14). The VNTRs were of different repeat lengths amongst different sculpin (n=4). The downstream 3' C-terminal region repeat consisted of short tandem repeats (STRs) with the trinucleotide (CCG) repeat unit (Figure 14). The STRs also varied in lengths between individual sculpin (n=3). This study is the first to report a nucleotide repeat in the NHE-3 of any animal.

Since long-horned sculpin live in a marine environment, the mechanism for acid-base regulation using the NHE-3 isoform would seem to be counter productive due to the estimated 10% influx of salt across the gill membrane epithelium (driven by the need to excrete H⁺ for pH regulation; Evans, 1984). The presence of NHE-3 in sculpin gill epithelium supports earlier findings showing the ability of these animals to maintain internal pH homeostasis during external and internal challenges (Claiborne, 2002). Recent studies have shown changes in expression of NHE-3 during metabolic stress in the gills of fresh water dace (Hirata, 2003). Dace NHE-3 expression increased following exposure of the animal to low pH water. In an initial test of the effect of metabolic acidosis on NHE-3 transcription, anesthetized sculpin were fitted with an intraperitoneal cannula and following overnight recovery (Claiborne, 1994), were infused with a bolus of 0.1N HCl or water (typically 5 ml; 2 mmol kg⁻¹) according to our well established

protocol (Claiborne, 1997). Fish were sacrificed at h 0.5, 2 and 4 hours post infusion (N=3 each for control and acidotic fish, for each time period) and gills processed as above. Following RNA isolation and Northern blotting (see methods section), an anti-sense probe for NHE-3 was used to visualize NHE-3 mRNA expression. Blots were stripped and incubated with labeled probe for sculpin ribosomal L8 (which has been used as an internal control for real-time PCR (Choe, K. P., *et. al.* 2004 and 2005) as a control for lane to lane RNA loading and quality. Northern detection of sculpin gill homogenates revealed a transcript at 4.3-4.8 kb in many of the samples, with the strongest signal from those animals subjected to the acidosis. This transcript size was similar to the dace (4.0 kb) (Harata, 2003) and rat (5.2 kb) (Bookstein, C., 1994) NHE-3 mRNA. The L8 bands were not detected in all samples either, but when apparent, were of the expected size (??? kb) in both control and experimental fish.

In fish measured 30 minutes after the acid infusion, all control fish and 2 of 3 experimental animals had similar L8 expression, indicating approximately equal total RNA loading. A strong band for NHE-3 was observed in one of the two acidotic fish, but only dim banding was detected in the control group. In the two hour group, Northern blots for all control and 2 of 3 experimental fish showed good RNA quality, and clear NHE3 expression was observed in both acidotic fish but very little was detected in controls. In the gill samples collected 4 hours post-infusion, the two acidotic fish with clear L8 expression also exhibited strong NHE3 bands. Unfortunately, the control samples for this time period showed little L8 so comparisons with controls could not be made.

Mean band densities were analyzed from the digital image of the Northern blots using public domain software (ImageJ 1.38v; National Institutes of Health, USA). NHE-

3 band density was normalized for total RNA quality by dividing the mean pixel density of the NHE-3 bands by the mean density of the L8 signal (when both were available). Using this approach, the mean normalized control measurements in the hour 2 group was 0.52 ± 0.01 vs. the acidotic fish 1.02 ± 0.07 ($p < 0.002$, mean \pm SE, N=3, and 2 respectively). Thus, the NHE-3 normalized expression was ~ two-fold higher following acid exposure. The small sample size precludes strong statistical analysis, but there is a clear trend of NHE-3 expression which is higher in the acid loaded fish versus the controls, especially at hour 2.

Importantly, measured *in vivo* net H⁺ efflux of acid following an identical acid infusion in this species reaches a maximum at ~hour 4 (Claiborne, 1997), so the observed change in NHE-3 message may indicate that alterations in mRNA expression and ultimately, protein synthesis of this antiporter, may play a role in the systemic regulation. This time course is also similar to the pattern which has been observed (using relative qPCR) in hagfish following acid infusion (Edwards, S. L., *et. al.* 2001). If an increase in transcription and synthesis of NHE-3 is partially responsible for the observed “whole animal” transfers, then up regulation of NHE-3 mRNA would be expected to occur following acid loading and prior to the peak of *in vivo* transfers.

In the mammalian kidney the NHE-2 and NHE-3 homologs work in tandem in salt reabsorption in the proximal tubules with the NHE-3 isoform expressed at higher levels. It is not known which NHEs are predominantly expressed in the marine teleost. Therefore, further work on NHE1-3 mRNA transcription is needed. To date, the sculpin is the only marine teleost for which NHE-2 and NHE-3 full length sequences have been described.

Interestingly, it is not known whether the trinucleotide repeats we found in the upstream 5' regions or the downstream 3' variable region effects transcription of the mRNA. Nucleotide repeats are not uncommon in the genome of organisms, but when located in mRNA, usually lead to changes in expression or complete loss of expression of the gene or associated genes (Lui, Z., *et. al.*, 2001). Nucleotide repeats may also be associated with genome duplication events in evolution. Studies have reported a large number of genes containing simple sequence repeat (SSR) in the brain of channel catfish. A homologue of the RAD23B gene was found to include trinucleotide repeats (CCG) within its coding region (Lui, Z., *et. al.*, 2001). Because of the importance of the RAD23B gene in the nucleotide excision repair (NER) system, the catfish RAD23B locus was further characterized. The (ACC) repeats encode a polythreonine (T) tract within the catfish RAD23B gene that is absent from the previously cloned human and mouse genes. Analysis of the allele variation at the locus indicated the existence of variable microsatellite repeats in the NER RAD23B gene, suggesting that the trinucleotide repeats are expanding or shrinking. The majority of catfish show 10 (ACC) repeats within the RAD23B gene, but alleles with 8 and 11 repeats were also detected. The (ACC) repeats are limited to only channel catfish and the closely related blue catfish, but are absent from flathead catfish and the cloned human and mouse genes, suggesting that the microsatellite invasion into the RAD23B gene is a recent event in evolution. The long-horned sculpin nucleotide repeat expansion only occurs in sculpin NHE-3, but does not occur in sculpin NHE-1 and NHE-2. Likewise, no repeats have been observed in stickleback genomic sequence data. It is possible that the role of sculpin NHE3 in acid base may be altered by this trinucleotide repeat phenomenon. Future studies will examine the function of the

long-horned sculpin NHE-3 in a mammalian expression system to determine if the trinucleotide repeat alters the kinetics of the NHE-3 transmembrane protein.

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Table 1: Primers list.

Primer Name	Nucleotide Sequence	Annealing Temp.
Sculp-NHE-2F	-TGTTTGCTTTTCGTATGGAGG-	
Sculp-NHE-2R	-TTNGCYTTNGGRTTNARNGGNGC-	
RB1 – NHE-3	-GTNCKYTGRAADATYTCYTG-	
RB2 – NHE-3	-CCATYTCNGGDATRAANGC-	
Fugu NHE-3-F1	-TCTGGGKTGGAACACKGGC-	51.0°C
Fugu NHE-3-F2	-TGGAACACKGGCTTCATCC-	47.0°C
Fugu NHE-3-F3	-GCNGWNGARATHATHAARGG-	44.0°C
Fugu NHE-3-R1	-AGGACTCMAGACGGCTYCTC-	51.0°C
Fugu NHE-3-R2	-TGYTGYTSNARNARRTGRTG-	45.0°C
Fugu NHE-3-R3	-YTTNTCYTCRAARTKYTTCC-	42.0°C
SculpNHE-3F	-CTTCATCCTCCTCNACGCTC-	
SculpNHE-3F-nested	-TGGACGGGAGCAAGATAAAG-	
SculpNHE-3R1	-TGATGTCCTCTGTTTCCCTC-	
F18NHE-3-F-DCE467	-TGAAACCTCTGGTCAACTGG-	49.7°C
F20NHE-3-F-DCE528	-AGGATTCTGATGAAGCCGTC-	50.7°C
F21NHE-3-F-DCE581	-AAGAAGTTCGGCGACGACTG-	54.4°C
B5NHE-3-R-DCE445	-TTCTCCTTTATCTTGCTCCC-	47.9°C
B1NHE-3-R-DCE415	-ATAAACTCCACGGGGACCAG-	52.9°C
B6NHE-3-R-DCE470	-CAGAGGTTTCATCGTCATTCC-	50.1°C
ScpNHE-3-176U24	-TTCCTCAAACATGGCAGCTACAAG-	60.7°C
ScpNHE-3-1003L23	-GGGGTCCACAGCAGCAATCAGAC-	60.7°C
ScpNHE-3-990U21	-CTTCTCTTCGGCAGTCTGATT-	57.3°C
ScpNHE-3-1850L22	-GAGTGTGAGCTCCGTCGGTGAA-	57.3°C
ScpNHE-3-1795U22	-TTCTCCAGGGAATGACGATGAA-	57.8°C
ScpNHE-3-2500L22	-TTCTTTGTGTGCTTGGAATCT-	57.8°C
ScpNHE-3-2414U19	-CGAGGTGCAGGAGATCTTC-	59.7°C
ScpNHE-3-2981L20	-GTGGGTGCTGCTACATGTGA-	59.7°C
ScpNHE-3-12U22	-CGAGGACACTGACATGGACTGA-	-----
ScpNHE-3-2985L20	-GGTGGTGGGTGCTGCTACAT-	-----
L8-F1	- GGATACACATCAAGGGAATCGTGAARGAYATHAT-	62.2°C
L8-F2	-GGGAATCGTGAAGGACATCATHCAYGAYCC-	61.1°C
L8-B1	-CAGTTTCGCTTGGCCTTGTAAYTTRTGRTA-	61.3°C
L8-B2	-CCGAAAGGGTGCTCCACNNGGRTTCAT-	60.2°C
GeneRacer TM 5'Primer	-CGACTGGAGCACGAGGACACTGA-	74.0°C
GeneRacer TM 5'Nested Primer	-GGACACTGACATGGACTGAAGGAGTA-	78.0°C
GeneRacer TM 3'Primer	-GCTGTCAACGATACGCTACGTAACG-	76.0°C
GeneRacer TM 3'Nested Primer	-CGCTACGTAACGGCATGACAGTG-	72.0°C

APPENDIX: LAB PROTOCOLS

Procedure for reverse transcription using Invitrogen Superscript II First-strand synthesis RT-PCR reaction.

- 1.) The total RNA needed for a single reaction in a total volume of 20 μ l was determined by the following formula

$$[\text{Absorbance} \times \text{dilution factor} \times 40 \text{ ng}/\mu\text{l} = \text{RNA concentration}]$$

- 2.) Mix and briefly centrifuge each component before use
- 3.) Prepare RNA primer master mix in sterile 0.2 or 0.5 ml tubes as follows

Component	Volume
Total RNA	X μ l
10 mM dNTP mix	1 μ l
Oligo (dT) ¹²⁻¹⁸ (0.5 μ g/ μ l)	1 μ l
DEPC treated water	X μ l
Total volume of single reaction primer mix	13 μ l

- 4.) The primer mix is incubated for 5 minutes @ 65°C
- 5.) The primer mix is chilled on ice for 2 minutes
- 6.) The following components are added to primer mix

10X RT buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	4 μ l
25 mM MgCl	2 μ l

- 7.) The mixture is incubated for 2 minutes at 42°C
- 8.) The following component is added to reaction mixture

Superscript TM II reverse transcriptase (50 units/ μ l)	1 μ l
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- 9.) The RT reaction mix is incubated for 50 minutes @ 42°C, 15 minutes @ 70°C and chilled on ice for 2 minutes to stop reaction

Protocol for RNA Formaldehyde gel electrophoresis

Preparation of 1.3% RNA Formaldehyde-Agarose Gel Protocol

Reagents:

20X MOPS buffer [400 mM 3-(N-morpholino)propanesulphonic acid, 160 mM sodium acetate, 20 mM EDTA, pH 7.0. Sterilize by autoclaving], Ethidium bromide (10 mg/ml)

in sterile DEPC treated water, Agarose, Formaldehyde, RNA loading buffer (50% glycerol, 0.1% bromophenolblue), 1X MOPS buffer [(ie, 20 mM MOPS, 8 mM sodium acetate and 1 mM EDTA (pH 7.0)], 7% (w/v) formaldehyde pH 4.0, 5% (v/v) sterile glycerol, 50% (v/v) deionized formamide
Protocol:

Preparation of agarose gel:

- 1.) Clean the gel-mold and comb with distilled water, then 70% ethanol. Seal the edges of the gel-former with tape. Check that the comb sits approximately 1 mm above the gel-mold when in situ
- 2.) Make up enough buffer for both the electrophoresis tank and the gel to avoid any differences in ionic strength between the two
- 3.) For 100 ml of a 1.3% gel: melt 1.3 g of agarose in 50 ml water. Add to 20 ml of formaldehyde 6, 5 ml of 20X MOPS and 5 ml of ethidium bromide solution in a measuring cylinder. Make the volume up to 100 ml with DEPC treated water
- 4.) Swirl to mix and then pour the gel carefully, checking for air bubbles under or between the comb
- 5.) The final gel should be between 3 mm and 5 mm thick. Allow it to set at room temperature for 30 - 45 minutes
- 6.) When fully set, pour enough buffer to cover the gel surface by ~ 1 mm and allow to stand for a couple of minutes
- 7.) Remove the comb carefully to avoid tearing the bottom of the wells (and subsequently losing the sample), and place the gel in the electrophoresis tank. Cover to a depth of ~ 1 mm

Prepare RNA samples:

- 1.) The loading buffer can be diluted by up to 2 fold although generally 5 ml of aqueous RNA is added to 15 ml of RNA loading buffer
- 2.) Denature the RNA samples in loading buffer by heating to 75°C for 10 minutes
- 3.) Chill on ice for 2 minutes before loading the gel

Electrophoresis of RNA sample:

Load the RNA samples carefully into the slots. Connect the electrophoresis tank to a constant voltage power supply - RNA will run from black to red (cathode to anode: make sure leads are on the right way round ! Run at 1 - 5V / cm (measured as the distance between the electrodes) until the RNA loading dye front has migrated the appropriate distance. Check after the dye has run 50% of the way down the gel. The ethidium bromide will migrate the opposite way to the RNA and long electrophoresis will remove much of the ethidium from the gel

Examination of RNA in agarose gel:

This relies on the UV-induced fluorescence of RNA intercalated ethidium bromide. However, formaldehyde fluoresces brightly when irradiated with UV light at 320 nm. Remove the formaldehyde by immersion in DEPC treated water - up to 6 changes over up to 2 hours. For a permanent record, photograph the gel when trans-illuminated by a 302 nm wavelength UV light source. This wavelength causes fluorescence of the intercalated ethidium bromide, but reduces the amount of 'UV nicking' of the RNA.

Protocol for PCR product subcloning using TOPO[®] TA cloning system

Reagents:

pCR[®]4-TOPO[®] [(10 ng/μl plasmid DNA in: 50% glycerol, 50 mM Tris-HCl, pH 7.4 (at 25°C), 1 mM EDTA, 2 mM DTT 0.1% Triton X-100, 100 μg/ml BSA and 30 μM phenol red)], Salt Solution (1.2 M NaCl, 0.06 M MgCl₂), DEPC treated water, SOC Medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose)], TOPO 10 F' cells, and LB plates containing 50 μg/ml kanamycin

Protocol:

Preparation of TOPO[®] Cloning reaction mix (6 μl) for transformation into competent TOPO 10 F' cells.

Components:	Volume:
PCR product	0.5 – 4 μl
Salt Solution	1 μl
DEPC treated Water	(add to a final volume of 5 μl)
TOPO [®] vector	1 μl
Total Volume	(6 μl)

Procedure:

- 1.) Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C)
- 2.) Place the reaction on ice and proceed to transforming competent cells or store the reaction overnight at -20°C
- 3.) Equilibrate a water bath to 42°C
- 4.) Warm the vial of SOC medium to room temperature
- 5.) Warm LB plates at 37°C for 30 minutes

- 6.) Thaw on ice 1 vial of TOPO 10 F' cells
- 7.) Add 2 μ l of the TOPO[®] cloning reaction into a vial of TOPO 10 F' cells and mix gently (do not pipette up and down)
- 8.) Incubate on ice for 5 to 30 minutes
- 9.) Heat-shock the cells for 30 seconds at 42°C without shaking
- 10.) Immediately transfer the tubes to ice
- 11.) Add 250 μ l of room temperature SOC medium
- 12.) Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour
- 13.) Spread 10-50 μ l from each transformation on a prewarmed selective plate and incubate overnight at 37°C.

Protocol of DNA gel electrophoresis

Procedure for DNA gel electrophoresis in 1 % agarose gel (50 ml volume)

Reagents:

10X Tris Borate EDTA (TBE) [(0.218 g Tris-base, 0.110 g Boric acid, and 9.3 g EDTA)], 10X DNA loading dye (20% glycerol, 0.1 M disodium EDTA pH, 1.0% SDS, and 0.25% bromophenol blue), ethidium bromide (see RNA gel electrophoresis)

*An alternative running buffer is Tris Acetate EDTA (TAE)

Protocol:

- 1.) Weigh out 0.5g of agarose into a 250mL conical flask. Add 50mL of 1X TBE , swirl to mix.
- 2.) Microwave for about 1 minute to dissolve the agarose
- 3.) Leave it to cool on the bench for 5 minutes down to about 60°C (just too hot to keep holding in bare hands).
- 4.) Add 1 μ L of ethidium bromide (10mg/mL) and swirl to mix

- 5.) Pour the gel into gel mold and leave to set for at least 30 minutes, preferably 1 hour
- 6.) Pour 1X TBE buffer into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer.
- 7.) Transfer an appropriate amount of each sample to a fresh microfuge tube.
- 8.) Add 0.2 volumes of loading buffer, (eg. 2µL into a 10µL sample). The tip will be used again to load the gel.
- 9.) Load the first well with DNA marker and Continue loading the samples

Close the gel tank, switch on the power-source and run the gel at 5V/cm

Stop the gel when the loading dye has run 3/4 the length of the gel

Switch off and unplug the gel tank and carry the gel (in its holder) to the dark-room to look at on the UV transilluminator

Protocol for Gel PCR product purification using BIO 101[®] System GeneClean[®] Spin Kit

Procedure for Gel DNA purification:

- 1.) Use sterile equipment to cut 300 mg agarose containing PCR product of interest from gel.
- 2.) Add 300 mg agarose to 400 µl GeneClean[®] Glass Milk 1.5 ml microcentrifuge tube.
- 3.) Heat mixture at 55 C until agarose melts (vortex).
- 4.) Centrifuge at 14,000 x g for 5-10 seconds or until liquid has emptied into the Catch Tube (empty Catch Tube as needed).
- 5.) Add 500 µl of New Wash solution to the filter
- 6.) Centrifuge at 14,000 x g for 5-10 seconds (discard flow through).
- 7.) Centrifuge at 14,000 x g 2 minutes to dry the pellet and transfer Spin Filter to fresh Catch tube.

- 8.) Add 15 μ l of Elution buffer to spin filter
- 9.) Carefully resuspend the pellet by gently pipetting up and down while stirring the pellet with the pipette tip. Centrifuge at 14,000 x g for 30 seconds to transfer eluted DNA to Catch Tube (Discard spin filter and cap the tube)